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GENOMIC SCIENCE PROGRAM PI MEETING ABSTRACTS

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U.S. Department of Energy Office of Science
Biological and Environmental Research Program
Biological Systems Science Division

2024 Genomic Science Program PI Meeting

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Bethesda, Md.

U.S. Department of Energy Biological and Environmental Research Program
Biological Systems Science Division

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About the Biological and Environmental Research program

The Biological and Environmental Research (BER) program supports transformative science and scientific user facilities examining complex biological, Earth, and environmental systems for clean energy and climate innovation. BER research seeks to understand the fundamental biological, biogeochemical, and physical principles needed to predict a continuum of processes occurring across scales, from molecules and genomes at the smallest scales to environmental and Earth system change at the largest scales. This research—conducted at universities, U.S. Department of Energy national laboratories, and research institutions across the country—is contributing to a future of reliable, resilient energy sources and evidence-based climate solutions.

About the Biological Systems Science Division

The Biological Systems Science Division (BSSD) seeks to understand, predict, manipulate, and design plant and microbial systems for advances in renewable energy, insights into environmental processes, and biotechnological breakthroughs supporting the U.S. bioeconomy. To expand knowledge of biological systems, BSSD supports basic research and capabilities in foundational genomic science, systems biology, genome engineering, computational analysis, molecular imaging, and structural characterization.

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2024 Genomic Science Program PI Meeting Abstracts

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BIOENERGY

Bioenergy Research Centers
Center for Advanced Bioenergy and Bioproducts Innovation (CABBI)

Beyond Boundaries: Foxtail Mosaic Virus Drives Heterologous Protein Expression and Precision Gene Editing in Sorghum

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Project Goals: The overall goal of this project is to develop viral vectors for delivering gene editing reagents and creating somatic and heritable mutations through infection in sorghum. Specifically, this work will:

- Engineer and optimize novel plant RNA viruses to express heterologous proteins and CRISPR-Cas gene editing reagents.
- Test systemic infections by tracking fluorescent protein "AmCyan" and determine the most effective plant RNA virus.
- Develop viral vectors with a large genome cargo size and the ability to infect meristem or germline cells. Determine the viral cargo capacities and the stability of viral cargo at mRNA and protein level.
- Develop a nontransgenic genome editing platform based on the engineered RNA virus vectors. Optimize novel viral vectors to express Cas9 endonucleases and gRNAs simultaneously from the viral genome.
- Test different transformation techniques (particle bombardment, agro-injection, viral sap rub inoculation) to co-inoculate several viruses to determine their synergistic interaction to increase meristematic viral infection and targeted mutagenesis frequencies.

Transformation is an important step in genome editing. The requirement for *in vitro* tissue culture and regeneration limits the technology's application to commercially relevant varieties of many crop species. To overcome this issue, plant viruses have recently been used as vectors for foreign gene expression, endogenous gene silencing, and delivering gene editing reagents to induce mutations mostly in Cas9-expressing transgenic plants, especially in dicots. However, a limited number of viruses have been developed into viral vectors for the purposes of gene editing in monocotyledonous plants. The team engineered a set of (monopartite) Foxtail Mosaic Virus (FoMV) and (tripartite) Barley Stripe Mosaic Virus (BSMV) vectors to deliver the fluorescent protein "AmCyan" to track viral infection and movement in *Sorghum bicolor*. Researchers further used these viruses to express and deliver single guide RNAs (sgRNAs) to Cas9-expressing transgenic sorghum lines, targeting *S. bicolor* Phytoene Desaturase (PDS), Magnesium-Chelatase (MgCh), and Lemon White (Lw) genes. BSMV was unable to infect sorghum and express "AmCyan" or deliver sgRNAs. In contrast, FoMV systemically infected the sorghum lines and induced somatic mutations at frequencies up to 60%, which produced phenotypes that were visibly distinguishable from the wildtype, indicating the potential applications of this virus for *in planta* gene editing and functional genomics studies in sorghum. Initial research indicates that FoMV vectors can be further engineered to gain the ability to induce mutations in the germline as well.

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Climate vs. Energy Security: Quantifying the Trade-Offs of BECCS Deployment and Overcoming Opportunity Costs on Set-Aside Land

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Project Goals: Bioenergy with carbon capture and storage (BECCS) is explicitly being put forth as a cost-effective strategy to reconcile negative emissions targets with a sustainable energy supply. The deployment of BECCS at scale, however, raises concerns over land displacement, compensatory agricultural expansion (indirect land use change; ILUC), and the derived toll on emissions savings. ILUC can be minimized by targeting energy feedstock production on set-aside land. However, whether energy feedstocks can be sourced without incurring self-defeating emissions from land conversion remains unclear. The first goal of this study was to evaluate the emissions cost of sidestepping the drawbacks of ILUC. In addition, at present there is an important mismatch between low-carbon (C) scenarios that rely on sustained negative emissions and the emissions reduction pledges that lead international climate action. Further, uncertainty in effective C removal rates and the political appeal of energy independence may make energy targets more marketable and prioritize ethanol yields in climate portfolios. Therefore, the second goal was to examine the tradeoffs of the strategic deployment of BECCS targeting these intimately linked yet inherently different priorities.

BECCS sits at the nexus of climate and energy security. The project evaluated tradeoffs between scenarios that support climate stabilization (e.g., negative emissions and net climate benefit) or energy security (e.g., ethanol production). Further, central to the sustainable deployment of BECCS, the team estimated the cost of sidestepping ILUC, including disturbance emissions (C debt) and foregone greenhouse gas emission savings from the displaced system (opportunity cost). The project's spatially explicit biogeochemical-life-cycle model indicates that the opportunity cost increased C emissions per unit of energy produced by 14 to 36%, roughly doubling breakeven times for the initial C debt and making geologic C capture and storage necessary to achieve negative emissions from any given energy crop. The toll of opportunity costs on the climate benefit of BECCS from set-aside land was offset through spatial allocation of crops based on their individual biophysical constraints. Dedicated energy crops consistently outperformed mixed grasslands. Researchers estimate that BECCS allocation to land enrolled in the Conservation Reserve Program (CRP) could capture up to 9 teragrams (Tg) of C per year from the atmosphere, deliver up to 16 TgC equivalent per year in emissions savings, and meet up to 10% of the U.S. energy statutory targets, but contributions varied substantially as the priority shifted from climate stabilization to energy provision. An energetically optimal deployment would generate 13.3 billion liters of ethanol annually but would reduce negative emissions by 21% and the net climate benefit of BECCS by 15% relative to alternative optimization strategies. Results indicate a significant potential to integrate energy security targets into sustainable pathways to climate stabilization but highlight the tradeoffs between divergent policy-driven agendas.

Blanc-Betes, E., et al. 2023. "Climate versus Energy Security: Quantifying the Trade-offs of BECCS Deployment and Overcoming Opportunity Costs on Set-Aside Land," *Environmental Science & Technology* **57**(48), 19732–48. DOI:10.1021/acs.est.3c05240.

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Metabolic Engineering of *Issatchenkia orientalis* for Cost-Effective Production of Citramalate

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Project Goals: This project aims to engineer a pH-tolerant strain, *Issatchenkia orientalis*, capable of using sustainable feedstocks to produce citramalate while achieving successful scale-up. Specifically, the team plans to:

- Increase citramalate titer and yield from glucose, feedstock hydrolysate, and oil through substrate optimization.
- Expand the knowledgebase and tool repertoire for *I. orientalis* by implementing advanced toolkits, such as optogenetics and piggyBac transposon technology, and conducting genetic studies by constructing a comprehensive knockout library.
- Conduct thorough investigations on scale-up conditions for the production process.

Methyl methacrylate (MMA) is a building block of poly MMA (PMMA), a material commonly recognized as acrylic glass or plexiglass. The prevailing method to manufacture PMMA utilizes petroleum and the acetone cyanohydrin process, which is considered unsustainable and raises concerns regarding the use of toxic chemicals. An alternative route using semisynthesis, combining converting microbially produced citramalate to methacrylic acid (MA) using a catalyst and final esterification of MA to MMA, could present a viable solution.

Previous studies have shown large titers of citramalate in engineered *Escherichia coli*. However, the increased production cost and carbon footprint from having a neutralization and reacidification step makes this route less economically attractive. A pH-tolerant strain such as *I. orientalis* would be an attractive alternative. Through extensive collaborative efforts, the Center for Advanced Bioenergy and Bioproducts Innovation (CABBI) team has previously demonstrated that *I. orientalis* can withstand citramalate of up to 80 g per liter at pH 3. Additionally, the team engineered a strain that produces 2 g/L citramalate by integrating the citramalate synthase gene (*cimA*) from *Methanocaldococcus jannaschii* into the *I. orientalis* genome using piggyBac.

Examining bottlenecks and increasing flux towards citramalate through metabolomic study and genome-scale modeling was essential in increasing citramalate production. The metabolomics studies found that there is pyruvate overflow, accumulation of intracellular citramalate, and excess ethanol byproduct. Using the genome-scale model, pathways of interest were identified to address these problems. The team simultaneously decreased pyruvate overflow and increased lacking acetyl-CoA by utilizing an aldehyde dehydrogenase gene (*ALD6*) from *Saccharomyces cerevisiae* and a mutated acetyl-CoA synthase gene (ACS_{SE}^{L641P}) from *Salmonella enterica*. Researchers also incorporated a multidrug transporter gene (*QDR3*) from *S. cerevisiae* to increase excretion of citramalate into the growing broth. Using piggyBac, researchers integrated *QDR3-ALD6-*ACS_{SE}^{L641P} and a more active *cimA* (*cimA3.7*) to create a library of variants. The team selected this library's top producer (Q42) for further engineering. To address excess ethanol production, the project employed CRISPR to delete the pyruvate decarboxylase gene (*PDC*). The resulting strain, Q42 Δpdc, produced 18 g/L of citramalate in shake flasks and was scaled up to a 3-L bioreactor to produce 30 g/L using synthetic complete medium with 6% ammonium sulfate, 5% glucose, and a trace metal supplement without needing pH or dissolved oxygen control.

In addition, the CABBI team successfully generated a xylose-utilizing strain, Q42X Δpdc, for future work involving potential feedstocks such as sorghum hydrolysate and sugarcane juice. Currently, the team is actively characterizing the metabolism and genomics of *I. orientalis* by developing a comprehensive knockout library and updating the genome-scale model. The team is also exploring new metabolic engineering strategies such as eliminating glycerol production, exploring alternative routes for acetyl-CoA production, identifying additional target genes for up- or down-regulation, and developing a light-controlled circuit.

Wu, Y., et al. 2023. "Metabolic Engineering of Low-pH-Tolerant Non-Model Yeast, *Issatchenkia* orientalis, for Production of Citramalate," *Metabolic Engineering Communications*. DOI:10.1016/j.mec.2023.e00220.

Funding Statement: This research was supported by CABBI, DOE, Office of Science, BER program, under award number DE-SC0018420.

High-Throughput Genome Editing and Phenotyping of Plant Cells Using a Scalable and Automated Pipeline

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Project Goals: This project aims to: (1) enhance bioproduct lipid production using CRISPR activation system in crops; (2) accelerate the design-build-test-learn plant bioengineering cycle through using a biofoundry (iBioFAB); and (3) improve high-throughput genome editing and metabolic engineering in crops to reduce labor and time during the process of plant bioengineering.

In plant synthetic biology, one challenge arises from the laborious and time-consuming bioengineering process. To address this, researchers established a fast, automated, scalable, and high-throughput pipeline for plant bioengineering (FAST-PB), which significantly, efficiently, and cost-effectively streamlines gene cloning, genotyping, and phenotyping processes. This pipeline not only enhances lipid production up to 6-fold, but also significantly accelerates the design-build-test-learn cycle in plant synthetic biology and facilitates plant regeneration. The other challenge lies in the fact that the synergistic fusion of biofoundry automation and single-cell MALDI has not been applied in plant research, which is a critical area for high-throughput metabolic engineering and cell biology. This study bridges this knowledge gap by successfully uniting automation techniques with MALDI methodology, enabling high-throughput single-cell lipid profiling. The pipeline demonstrates high versatility, with main applications in synthetic biology, genome editing, metabolic engineering, single-cell metabolomics, and plant regeneration.

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Comparison of Soil Responses to Long-Term Fertilization and Short-Term Nitrogen and Carbon Amendments in Miscanthus and Corn

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Project Goals: This work aims to shed light on the impacts of long-term and short-term nitrogen (N) inputs and of *Miscanthus* x *giganteus* (miscanthus) on soil responses. Greenhouse gas emissions, net nitrogen mineralization, and microbial community nitrogen cycling genes were compared between miscanthus and maize soils with varying legacies of N fertilization and with contemporary N amendments.

Understanding the role of bioenergy crops in carbon and nitrogen dynamics is crucial for sustainable production of biofuels and bioproducts. Miscanthus, a promising perennial biomass crop, is favored due to its high biomass yields compared to annual crops like maize. However, the effects of miscanthus on carbon sequestration and reducing nitrogen leaching and emissions compared to corn have been inconsistent. In this study, the team directly compared soils from miscanthus and maize fields for greenhouse gas emissions, net nitrogen mineralization, and the abundance of microbial nitrogen cycling genes over a 150-day soil incubation period. Soils were obtained from miscanthus and corn fields at the end of the growing season for incubation experiments. The team evaluated these incubation soil responses to compare the impacts of legacies of fertilization to the responses to contemporary amendments. The results revealed that cumulative soil nitrous oxide (N_2O) emissions increased during the incubation, with miscanthus producing significantly greater N_2O than corn. Additionally, higher fertilization levels resulted in greater N_2O production, with N amendment showing a larger effect than C amendment. Net N mineralization was significantly affected by crop type and amendment but not historical fertilization. Microbial processes play a crucial role in determining soil N availability. The team observed no significant differences in total copies of the 16S rRNA gene between crops, historical fertilization treatments, or amendments. However, the abundance of specific bacterial genes involved in N cycling varied, with higher copies of genes associated with nitrification in miscanthus soils and an increase with historical fertilization levels. Genes encoding nitric oxide reductases generally

decreased with higher N fertilization levels. The team found that contemporary N addition increased N_2O production as expected, but the larger difference in N_2O production was explained by the legacy of fertilization. This trend was observed in both corn and miscanthus but significantly supported only in miscanthus, indicating a unique response of miscanthus to fertilization legacy. Overall, the results suggest that microbial processes in miscanthus soils differ significantly from maize and emphasize the importance of considering previous land management history when evaluating the contribution of miscanthus and bioenergy crops to N balances.

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Machine Learning–Assisted Genome-Wide Association Study Uncovers Copy-Number Variations of Tandem Paralogs Driving Stress Tolerance Evolution in *Issatchenckia orientalis*

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https://cabbi.bio/

Project Goals: This project aims to exploit the genetic and phenotypic diversity of *Issatchenckia orientalis* through population genomics and machine learning approaches to: (1) explore the potential molecular mechanisms behind its multi-stress tolerance; (2) develop predictive models for various stress tolerances; and (3) identify key genes associated with tolerance to industrial stresses and resistance to different fungicides.

The environmental yeast *I. orientalis* plays a dual role in human society, owing to its multi-stress tolerance. Its ability to withstand common industrial stressors, such as low pH and high temperatures, makes it an ideal candidate for engineered biosynthesis of bioproducts. However, it also poses significant health risks as a multidrug-resistant pathogen capable of causing invasive fungal diseases and is recognized by the World Health Organization as a priority fungal pathogen. Deciphering the molecular mechanisms and evolutionary paths of its stress tolerance is key for managing risks and exploiting the benefits of this species. Here, researchers report the potential mechanisms driving the adaptive evolution of *I. orientalis* to various stressors using population genomics and machine learning approaches.

Researchers conducted whole-genome sequencing of 170 *I. orientalis* isolates and assessed the growth of 161 isolates under 57 different stressors, including heat, low pH, organic acids, lignocellulosic inhibitors, and fungicides from different families (e.g., Azoles, Polyenes, and Echinocandins). The team also developed a machine-learning-assisted analysis pipeline, Machine-Learning-Assisted Engineering of Stress-Tolerance Rational Optimization (MAESTRO), to streamline the analysis. MAESTRO revealed that pleiotropic effects of copy-number variations (CNVs) among a small set of genes (less than 3.5%) play a significant role in *I. orientalis'* stress tolerance. Using CNVs as features, the team successfully correlated genetic variation with phenotypic variation of stress tolerance, demonstrating a median coefficient of determination (R^2) of 0.67 and a median Pearson's correlation of 0.92 across 57 stress conditions when comparing actual fitness to predicted fitness values. Notably, many of these genes (17 to 23%) were tandem repeat paralogs (TRPs), a genomic configuration known as hot spots for gene amplification, reduction, or even shuffling to invent new activities. Additionally, TRPs were significantly enriched with transporters (52%, compared to the genome-wide average of 3%), likely composing the resistome network. Finally, as a proof of concept, the team engineered a strain with enhanced tolerance to the lignocellulosic inhibitor 5-hydroxymethyl furfural but lower resistance to the fungicide fluconazole by deleting four TRPs. This work demonstrates the potential of leveraging fungal genetic variation to predict their potential risks in society and designing more robust industrial strains to develop a sustainable bioeconomy.

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Progress Towards the Generation of Oily Miscanthus

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https://cabbi.bio/

Project Goals: The goal of this project is to increase the production of vegetative lipids in miscanthus via genetic engineering.

Miscanthus, a Center for Advanced Bioenergy and Bioproducts Innovation (CABBI) feedstock, is a tall perennial rhizomatous temperate grass. Because of its high biomass yield, high carbohydrate and low ash content, high calorific value, remarkable environmental adaptability, high water and land use efficiency, and low fertilizer and pesticide requirements, it has become one of the most promising sustainable crops for biomass, bioenergy, and bioproducts. One of CABBI's major goals is to increase the value of its feedstocks by using the "plants as factories" paradigm to produce novel high value bioproducts. Previously, researchers have demonstrated the ability to transform and edit multiple lines in the two major species of miscanthus, *Miscanthus sinensis* and *M. sacchariflorus*, and a high-performing ortho-species *M.* x *giganteus*, which is a hybrid of the two species. Here, the team reports the progress of this research on transforming miscanthus with two constructs designed to increase vegetative triacylglycerol production. These constructs, pPTN1569 and pPTN1586, were previously vetted in sorghum.

Trieu, A., et al. 2022. "Transformation and Gene Editing in the Bioenergy Grass Miscanthus," *Biotechnology for Biofuels and Bioproducts* **15**. DOI:10.1186/s13068-022-02241-8.

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Bioenergy Research Centers
Center for Bioenergy Innovation (CBI)

Knocking Out a Candidate Gene for Wax Production in Switchgrass Results in an Unexpected Pleiotropic Phenotype

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https://cbi.ornl.gov

Project Goals: The Center for Bioenergy Innovation's (CBI) vision is to accelerate domestication of bioenergy-relevant, nonmodel plants and microbes to enable high-impact innovations along the bioenergy and bioproduct supply chain while focusing on sustainable aviation fuels (SAF). CBI has four overarching innovation targets: (1) develop sustainable, process-advantaged biomass feedstocks; (2) refine consolidated bioprocessing with co-treatment to create fermentation intermediates; (3) advance lignin valorization for biobased products and aviation fuel feedstocks; and (4) improve catalytic upgrading for SAF blendstocks certification.

Switchgrass, *Panicum virgatum*, a grass native to North America, is of intense interest as a dedicated feedstock for the production of SAF. Switchgrass ecotypes differ by a number of characteristics, including the presence of wax on leaves and stems. Lowland ecotypes generally contain high levels of C33 β-diketones and hydroxy-β-diketones, which are associated with the formation of crystalline wax tubes on the abaxial leaf side and a blueish plant color (Bragg et al. 2020; Weaver et al. 2018). In contrast, β-diketones are largely lacking from upland accessions, which therefore have glossy green leaves. Researchers previously identified a cluster of genes as strong candidates for the quantitative trait locus that was identified for wax variation in an F_2 population from a cross between the lowland genotype AP13 and the upland genotype VS16 (Qi et al. 2021). One of the candidate genes, a likely 3-ketoacyl-CoA synthase 5 (KCS-5), was knocked out in Performer7, a transformable lowland accession, using CRISPR-Cas9. Interestingly, while edited plants had the expected glossy green color, they were also shorter in stature and had more tillers compared to the controls. To determine the effect of the KCS-5 knockout on transcription, an RNA-seq analysis was conducted on two independent KCS-5 knockout plants and two unedited control plants. A total of 1,781 and 415 genes were differentially expressed (DE) in leaves and stems, respectively, between the KCS-5 knockout lines and unedited controls (p-value≤0.05, log2-fold difference≥1). Of the DE genes in stems, 64% were also DE in leaves. Work is ongoing to determine the affected pathways as well as the effects of the KCS-5 knockout on sustainability.

Bragg, J., et al. 2020. "Environmentally Responsive QTL Controlling Surface Wax Load in Switchgrass," *Theoretical and Applied Genetics* **133**, 3119–37. DOI:10.1007/s00122-020-03659-0.

Qi, P., et al. 2021. "Quantitative Trait Locus Mapping Combined with Variant and Transcriptome Analyses Identifies a Cluster of Gene Candidates Underlying the Variation in Leaf Wax Between Upland and Lowland Switchgrass Ecotypes," *Theoretical and Applied Genetics* **134**, 1957–75. DOI:10.1007/s00122-021-03798-y.

Weaver, J. M., et al. 2018. "Cuticular Wax Variants in a Population of Switchgrass (*Panicum virgatum* L.)," *Industrial Crops and Products* **117**, 310–16. DOI:10.1016/j.indcrop.2018.02.081.

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Thermophilic Genetic Tool Development for Engineering and Functional Genomics in *Clostridium thermocellum*

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Project Goals: The Center for Bioenergy Innovation (CBI) vision is to accelerate domestication of bioenergy-relevant, nonmodel plants and microbes to enable high-impact innovations along the bioenergy and bioproduct supply chain while focusing on sustainable aviation fuels (SAF). CBI has four overarching innovation targets: (1) develop sustainable, process-advantaged biomass feedstocks; (2) refine consolidated bioprocessing with cotreatment to create fermentation intermediates; (3) advance lignin valorization for bio-based products and aviation fuel feedstocks; and (4) improve catalytic upgrading for SAF blendstocks certification.

Domestication and genetic engineering of nonmodel plants and microbes for optimal bioconversion performance is essential to achieve efficient and sustainable production of fuels and chemicals from lignocellulose. *Clostridium thermocellum* is an anaerobic thermophile capable of directly degrading lignocellulose to fermentable sugars, which eliminated the additional step of chemical or enzymatic deconstruction of lignocellulosic biomass and mediating cost-effective consolidated bioprocessing (CBP) by also fermenting the sugars into intermediates (e.g., ethanol). While *C. thermocellum* has powerful capabilities in biomass solubilization, further strain improvement is needed for yield, titer, and selectivity. Available genetic engineering tools for *C. thermocellum* were limited by inefficient conventional homologous recombination. However, recent work introduced two alternative, efficient, and timesaving CRISPR-Cas genome editing systems utilizing the native Type I-B from *C. thermocellum* and the heterologous Type II CRISPR system from *Geobacillus stearothermophilus*, with Lambda Red exo/beta homologs from *Acidithiobacillus caldus* for RecA-independent homologous recombination (Marcano-Velazquez et al. 2019). In addition to continued efforts to improve transformation efficiency through plasmid methylation platforms, researchers are developing genetic tools towards high-throughput genome-scale studies to enable gene-to-trait identification. Traditional homologous recombination in *C. thermocellum* is challenging and time-consuming, especially for chromosomal insertion of heterologous genes. To enable faster and easier heterologous gene insertion in *C. thermocellum*, researchers developed a thermostable version of the Serine recombinase Assisted Genome Engineering (tSAGE) technique using the recombinase from *Geobacillus* sp. Y412MC61 (Walker et al 2020). Using the tSAGE method to integrate the tested genetic elements, researchers tested 15 homologous and 32 heterologous promoters, four inducible promoters, five riboswitches, 10 fluorescent reporter genes, and a library of gene cassettes with variable distances between the ribosome binding site and the start codon of the promoter. The tSAGE method allowed researchers to quickly screen and identify the genetic elements that will improve strain engineering for CBP of lignocellulose into sustainable fuels and chemicals. tSAGE will also be key to efficiently screen modified metabolic pathways in C. *thermocellum*.

The CRISPR-Cas genome editing tools are useful for permanent genetic engineering such as deletions and insertions. To enable temporary perturbations in gene expression, researchers repurposed the native Type I-B CRISPR system and generated a catalytically dead CRISPR system capable of CRISPR-interference (CRISPRi) and gene knockdown by deleting the Cas3 nuclease. To achieve reversibility and temporal control in gene knockdowns, the team identified a xylose-inducible promoter and a 2-aminopurine-inducible riboswitch (Yang et al. 2014) that function efficiently in a dose-dependent manner in *C. thermocellum*. Researchers are currently working on generating a *C. thermocellum* strain capable of inducible CRISPRi by integrating the inducible gene expression systems into its genome to drive the expression of the Cas3-deleted CRISPRi system. The reversibility and temporal control of the inducible CRISPRi system will allow rapid and high-throughput genome-wide screens for genotype-phenotype discovery and enable identification of new gene function in *C. thermocellum*. The inducible gene expression systems identified in this work will also be useful to control gene expression for engineering pathways and optimizing CBP of lignocellulose into ethanol in *C. thermocellum*.

Marcano-Velazquez, J. G., et al. 2019. "Developing Riboswitch-Mediated Gene Regulatory Controls in Thermophilic Bacteria," *ACS Synthetic Biology* **8**(4), 633–40.

Walker, J. E., et al. 2020. "Development of Both Type I–B and Type II CRISPR/Cas Genome Editing Systems in the Cellulolytic Bacterium *Clostridium thermocellum*," *Metabolic Engineering Communications* **10**, e00116.

Yang, L., et al. 2014. "Permanent Genetic Memory with >1-Byte Capacity," *Nature Methods* **11**(12), 1261–66.

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Intraspecific Genetic Variation in *Populus trichocarpa* Influences Above and Belowground Plant Chemistry and Influences Plant–Soil Interactions

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Gaining a predictive understanding of above- and belowground plant system performance is critical to developing climate-smart and sustainable bioenergy crops for SAF. Plant and soil (including microbial) interactions are known to influence nutrient and water uptake, microbial association, biomass productivity, climate adaptability, and soil carbon storage. Current understanding of variability and correlations among aboveground properties of bioenergy crops and their underlying genetics is far greater in comparison to that of the belowground properties (root, soil, and microbial), which is explained by data asymmetries between above- and belowground datasets. The challenges of laborious sampling, lack of standardized methods, and sparse measurements are further confounding to available belowground empirical data. To achieve a holistic understanding of bioenergy crop performance, researchers are undertaking laboratory- and field-based belowground performance evaluations of natural genotypes of the perennial woody bioenergy crop species *Populus trichocarpa.* This project presents the results from two such studies, one at greenhouse scale and another at field scale.

The utility of genome-wide association study (GWAS) analysis in identifying single nucleotide polymorphisms (SNPs) associated with variation in traits of interest in *Populus* has been previously demonstrated with remarkable genomic resolution for aboveground traits such as biomass yield, phenology, and lignin content (Evans et al. 2014; Xie et al. 2018). There has been limited progress, however, in defining the genetic components of belowground root-related traits. Researchers undertook a GWAS of greenhouse root chemistry and coassessment of below- and aboveground traits. The results showed that above- and belowground chemistry is largely independently controlled and provided the first comprehensive collection of genes and SNPs associated with variation in root chemistry of a bioenergy crop species. Furthermore, the team identified a subset of SNPs and genotypes that are coevolved in trait combinations potentially favorable to aboveground valorization of biomass and belowground storage of carbon (C). The discovery of both colocated or independent SNPs and naturally superior genotypes from the analyses serve as high-potential springboard levers in advanced synthetic biology and breeding strategies for desirable below- and aboveground traits.

Researchers undertook a pilot empirical evaluation study to inform a planned population-wide sampling effort and iterative modeling designed to understand plant–soil interactions in a mature, common garden stand of the same GWAS population in the Pacific Northwest. This study, using four different *P. trichocarpa* genotypes (population extremes in lignin content), demonstrates the value of leveraging mature GWAS common garden sites and the potential of focal tree soil sampling for detecting genotype-specific associations with soil biogeochemistry [e.g., C, nitrogen (N), phosphorus (P), and a range of soil micro- and macronutrient elements] and soil bulk density, as well as the extent of correlations between above- and belowground traits. These analyses revealed positive relationships between root C and soil C; root C:N and soil calcium; and a negative relationship of soil C with soil pH, K, and P. In the future, expansion of such species-, age-, and region-specific efforts will enhance predictability of plant–microbe–soil interactions in bioenergy crop systems. This will ultimately accelerate the identification of genetic and edaphic factors that can enhance bioproduct yield, C storage, and sustainability.

Evans L.M., et al. 2014. "Population Genomics of Populus Trichocarpa Identifies Signatures of Selection and Adaptive Trait Associations," *Nature Genetics* **46**(10), 1089–96.

Xie M., et al. 2018. "A 5-Enolpyruvylshikimate 3-Phosphate Synthase Functions as a Transcriptional Repressor in Populus," *Plant Cell* **30**(7), 1645–60.

Funding Statement: Funding was provided by the Center for Bioenergy Innovation (CBI) led by Oak Ridge National Laboratory (ORNL). CBI is funded as a DOE Bioenergy Research Centers supported by the BER program in the DOE Office of Science under FWP ERKP886. ORNL is managed by UT-Battelle, LLC, for DOE under contract number DE-AC05-00OR22725.

Quantitative Trait Locus Mapping of Swarming Motility and Germination Rate in a *Bacillus subtilis* Library

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Overview: Linking genes of unknown function to relevant phenotypes in microbial systems is challenging. This necessitates the development of novel bacterial quantitative trait loci (QTL) mapping techniques enabled by genome shuffling. *Bacillus subtilis* is used as a proof-of-concept model for phenotyping and mapping causal genetic variants. Novel hardware and software were developed for high-throughput phenotyping, including an XY-robot for automated imaging and mathematical and statistical methods for image processing and feature extraction. Genome-wide association studies (GWAS) based on methods used in plant populations were used to identify predictive loci and validate causative genetic variants.

Approach: The approach is broken into five steps: (1) genome shuffling by protoplast fusion mimics sexual recombination in bacteria to enable QTL mapping (Vasileva et al. 2022); (2) a custom-built XY-robot with camera modularity and automated sample tracking enables high-throughput imaging; (3) convolutional neural networks (Lagergren et al. 2023) and mathematical models are used to predict morphological features of swarm assays and spore gemination dynamics; (4) genomic analysis is used to map multiple swarming and germination phenotypes using the QTL population; and (5) new strains are created with targeted modifications to confirm that genes in the QTL are causal.

Results: This work demonstrated *B. subtilis* as a proof-of-concept model to generate 386 recombined strains with 15,906 variants. The custom XY-robot captured high-resolution images of microorganisms in six-well plates at 20 to 30 images per minute. Mathematical and statistical models extracted multiple phenotypes relating to swarming motility and germination. Novel software was developed to extract gene regions for alignment-based sample binning across the population. Genomic analysis revealed highly significant SNPs associated with colony area and germination. Causal regions were validated through the creation of new *B. subtilis* strains with targeted modifications that exhibited phenotypic differences in, for example, spore germination.

Impact: After successful demonstration in *B. subtilis*, researchers are now applying the same approach in *Clostridium thermocellum*, a model system relevant to bioprocessing and enzyme engineering. A similar approach could be widely used to connect phenotype to genotype in other bacteria important for the bioeconomy.

Vasileva, D., et al. 2022. "Protoplast Fusion in *Bacillus* Species Produces Frequent, Unbiased, Genome-Wide Homologous Recombination," *Nucleic Acids Research* **50**(11), 6211–23.

Lagergren, J., et al. 2023. "Few-Shot Learning Enables Population-Scale Analysis of Leaf Traits in *Populus trichocarpa*," *Plant Phenomics* **5**(75).

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Global Proteomics and Resource Allocation Modeling Reveals Thermodynamic Bottleneck and Highlights Effective Genetic and Metabolic Interventions for *C. thermocellum*

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Consolidated bioprocessing (CBP) with *Clostridium thermocellum* is a promising route to produce renewable biochemicals (such as ethanol) from lignocellulosic biomass. A disadvantage of the organism is the relatively low thermodynamic driving force (TDF) through its glycolysis, which limits ethanol titer, as less favorable energy substrates (e.g., pyrophosphate instead of adenosine triphosphate) or metabolic pathways (such as the malate shunt) are used. Recently, an improved genome scale metabolic model (GSMM) was developed to help highlight potential energetic solutions in *C. thermocellum* (Schroeder et al. 2023). This presentation highlights recent efforts to quantify the proteomic and metabolic cost of low TDF and proposes interventions minimizing proteomic cost while maximizing TDF. Absolute concentration of 18 proteins and relative protein concentration (iBAQ) were measured under cellobiose-limed chemostat growth conditions. The correlation between absolute and iBAQ values were used to estimate absolute concentration for each measured protein. Enolase was discovered to be the enzyme with the greatest number of copies per cell, with more than 3.3 times that of the next most abundant enzyme accounting for more than 14% of estimated total protein mass per cell. Enolase has been previously shown to occur at the end of a series of four near-equilibrium reactions, the enzyme of which accounts for nearly 20% of total protein mass per cell, suggesting *C. thermocellum* uses a "pull" approach through these steps and is a key target for both improving TDF and reducing protein burden. To evaluate potential interventions, a resource allocation model (RAM) of *C. thermocellum* was reconstructed from the proteomics data and the recent stoichiometric GSMM model. The RAM was used to quantify proteomic and metabolomic effects of several intervention strategies including improving TDF through these steps, creating a greater "push" effect, and creating a greater "pull" effect. This allows researchers to test possible metabolic solutions *in silico*, prior to experimental validation via genetic engineering.

Schroeder, W. L., et al. 2023. "A Detailed Genome-Scale Metabolic Model of *Clostridium thermocellum* Investigates Sources of Pyrophosphate for Driving Glycolysis," *Metabolic Engineering* **77**, 306–22. DOI:10.1016/j.ymben.2023.04.003.

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Optimizing Biological Funneling of Lignin Streams by Comparison in Several Microbial Platforms

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Lignin is a complex aromatic polymer found in plant cell walls and accordingly represents an underutilized, but also recalcitrant, carbon-rich stream in lignocellulosic biorefineries. Processes to valorize lignin to high-value products are therefore of interest. Biological funneling of the heterogeneous lignin-related compounds (LRCs) generated by chemical or enzymatic deconstruction into performance-advantaged products is a promising strategy toward this goal (Rinaldi et al. 2016; Sun et al. 2018). This project compares the catabolic capacities of several microbial platforms for biological funneling and identifies metabolic inefficiencies in the production of muconic acid from LRCs.

To characterize catabolic capabilities of several promising microbial strains as hosts for the valorization of lignin streams, growth, and substrate utilization of six bacterial strains and one yeast were directly compared on representative LRCs. *Pseudomonas putida* bacteria exhibited the fastest growth rate, highest tolerance, and broadest substrate range when grown on a lignin-rich stream, a model aromatic LRC mixture, guaiacyl (G)-type compounds, *p*-coumaryl (H)-type compounds, and aliphatic acids. *Sphingobium lignivorans* bacterial strains utilized the highest concentration of syringyl (S)-type compounds. This work provides a foundational comparison of microbial platforms for LRC catabolism as well as genetic reserves to tap for unique metabolic capabilities.

Systems-level characterization of muconic acid production from aromatic LRCs and biomass production from glucose was conducted in *P. putida* to identify metabolic inefficiencies and bottlenecks. After rewiring native cellular metabolism of 4-hydroxybenzoate to muconic acid in the production strain *P. putida* CJ781 (CJ781; Kuatsjah et al. 2022), a bottleneck was identified at the catechol 1,2-dioxygenase. Proteomics, exometabolomics, and fluxomics analyses of glucose conversion to biomass growth and energy revealed that, relative to the wildtype strain, CJ781 exhibited greater secretions of intracellular metabolites, higher periplasmic flux, and increased ATP production. Notably, CJ781 secreted pyruvate and acetate, indicating a potential bottleneck in carbon flux entering the tricarboxylic acid cycle. Together, this work improves understanding of divided cellular metabolism between product formation and biomass production and identifies nonintuitive genetic targets for optimization of biological funneling.

Kuatsjah, E., et al. 2022. "Debottlenecking 4-Hydroxybenzoate Hydroxylation in *Pseudomonas putida* Kt2440 Improves Muconate Productivity from P-Coumarate," *Metabolic Engineering* **70**, 31–42.

Rinaldi, R., et al. 2016. "Paving the Way for Lignin Valorisation: Recent Advances in Bioengineering, Biorefining, and Catalysis," *Angewandte Chemie International Edition* **55**, 8164–215.

Sun, Z., et al. 2018. "Bright Side of Lignin Depolymerization: Toward New Platform Chemicals," *Chemical Reviews*, **118**(2), 614–78.

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Utilizing Cryo-Electron Microscopy to Characterize Proteins Relevant to Biomass Biosynthesis and Bioconversion

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The degradation of plant material via bacterial digestion to be converted into bioproducts such as ethanol is a main CBI focus. Plant cell walls are comprised of cellulose, hemicellulose, and lignin, which combine to make the cell wall recalcitrant to total digestion. Historically, cryo-electron microscopy (cryo-EM) was essential to confirming the structure of the cellulose synthase rosette. Solving these problems requires deeper understanding of both the biosynthesis pathways to make recalcitrant biomass polymers and the conversion pathways to break down biomass. Here, the research team utilized cryo-EM to further delve into key enzymes and complexes, with the aim of understanding biomass biosynthesis and bioconversion at a molecular level.

The first target of this work is to investigate the poorly explored proteins necessary for hemicellulose formation, which has a complex branching pattern. Most hemicellulose synthesis occurs in the Golgi apparatus in a non-templated manner, meaning that the branching sugar chains are added to the main xylan backbone presumably due to local protein interaction networks (Chou et al. 2015). By examining the near-atomic structures of glycosyltransferase proteins as determined by cryo-EM, both alone and in complex, the molecular mechanisms of hemicellulose synthesis and branching can be determined. The aim is to modulate the pathways to create less recalcitrant plants that remain robust and grow rapidly (Smith et al. 2022).

Another target for plant recalcitrance is to examine the digesting bacterium. *Clostridium thermocellum* is one of the best bioprocessing organisms identified to date. However, it is hampered in its ability to produce industrially relevant titers of bioproducts, such as ethanol. In past directed evolution studies regarding ethanol tolerance in *C. thermocellum*, one of the most frequently mutated proteins was AdhE, an alcohol-aldehyde dehydrogenase that produces ethanol. AdhE forms fascinating, spring-like ultrastructures that contain up to 100 AdhE monomers. Using cryo-EM, the research team solved the highest resolution structure of the AdhE ultrastructure to date, providing insight into the protein's catalytic pockets, as well as furthering understanding of intermediate aldehyde channeling (Ziegler et al. 2024). The results from the cryo-EM structure are feeding directly into mutagenesis studies to increase *C. thermocellum* ethanol production and tolerance.

Chou, Y-H., et al. 2015. "Protein–Protein Interactions Among Xyloglucan-Synthesizing Enzymes and Formation of Golgi-Localized Multiprotein Complexes," *Plant and Cell Physiology* **56**(2), 255–67.

Smith, P. J., et al. 2022. "Enzymatic Synthesis of Xylan Microparticles with Tunable Morphologies," *ACS Materials Au* **2**(4), 440–52.

Ziegler, S. J., et al. 2024. "Structural Characterization and Dynamics of AdhE Ultrastructures from *Clostridium thermocellum*: A Containment Strategy for Toxic Intermediates?" *bioRxiv*. DOI:10.1101/2024.02.16.580662.

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Bioenergy Research Centers
Great Lakes Bioenergy Research Center (GLBRC)

Combinatorial Library Design for Improving Isobutanol Production in *Saccharomyces cerevisiae*

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Project Goals: Improvement of isobutanol production in the yeast *Saccharomyces cerevisiae* by screening a combinatorial library of isobutanol pathway genes.

The branched-chain alcohol isobutanol absorbs less water and has a higher energy density than ethanol, making it a promising next-generation biofuel. Isobutanol can also be catalytically upgraded to produce sustainable aviation fuel. The yeast *Saccharomyces cerevisiae* is well-suited for bioproduction of isobutanol given its tolerance to stressors, fast growth rate, and extensive genetic toolkit. However, the native, robust ethanol fermentation of *S. cerevisiae* hinders isobutanol production. To increase isobutanol production in *S. cerevisiae*, researchers created a combinatorial library from a diverse set of isobutanol pathway genes. For each of the five enzymatic reactions in the isobutanol pathway, the library varied both enzyme variant and promoter strength, for a combined library size of ~1 billion unique members. Though its large size prevented comprehensive screening, researchers identified several sets of genes enabling high isobutanol production. These genes were then inserted into a strain of *S. cerevisiae* that cannot produce ethanol. The resulting strains produced isobutanol at yields close to the highest reported in academic literature, without producing ethanol. The inability to completely screen this library led researchers to design and assemble a smaller library of just the first three genes in the isobutanol pathway. This new library will be screened completely by using a combination of growth coupling and a *S. cerevisiae* strain containing a fluorescent biosensor for isopentanol, which shares a key intermediate compound with the isobutanol pathway. Next-generation sequencing of the enriched library will reveal further insights into which gene combinations from the library are best for isobutanol production.

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Engineering Inducible Biological Nitrogen Fixation for Bioenergy Crops

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Project Goals: This project aims to reduce bioenergy production's dependence on synthetic nitrogen fertilizers by promoting biological nitrogen fixation in bioenergy crops. Bacteria typically perform nitrogen fixation under low nitrogen and low oxygen conditions. Various genetic strategies have been developed to deregulate the system and enable nitrogen-fixing bacteria (diazotrophs) to fix nitrogen constitutively. However, nitrogen fixation is an energy-intensive process, and these manipulations reduce the fitness of engineered diazotrophs, making them less competitive than native ones. To overcome this challenge, researchers are engineering an inducible nitrogen fixation system that activates when diazotrophs can sense molecular signals from plant roots. Using plant molecules found in root exudates as signals and various biosensors, researchers aim to trigger nitrogen fixation in an inducible manner and optimize the delivery of fixed nitrogen to bioenergy crops.

Microbes can provide many benefits to plants, including plant growth promotion, enhancement of plant immunity, and nutrient uptake. In particular, some bacteria possessing a nitrogenase enzyme (diazotrophs) can convert atmospheric nitrogen into ammonium in a process known as biological nitrogen fixation. However, providing these benefits has a fitness cost for the diazotrophs. Nitrogen fixation, for instance, is well known to be very energetically expensive, making it a highly regulated process. Consequently,

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diazotrophs mostly fix nitrogen only under low nitrogen and low oxygen conditions. In gamma-proteobacteria, the NifL protein responds to environmental nitrogen and represses the expression of NifA, which is the master activator of the nitrogenase gene cluster. Disrupting the *nif*L gene and activating *nif*A is a classical strategy to enhance nitrogen fixation and trigger ammonium excretion, but this manipulation reduces the fitness of engineered bacteria. The project aims to engineer diazotrophs associated with plants with inducible biosensors that would enhance nitrogen fixation and ammonium excretion only in the presence of the host plant. The team isolated *Klebsiella variicola* and *K. michiganensis* strains from sorghum and maize roots. They are excellent nitrogen fixers, non-pathogenic, and tractable, making them suitable candidates for genetic engineering.

The team replaced *nif*L with an arabinose-inducible biosensor to drive the expression of *nif*A, thus activating the nitrogenase activity in the presence of arabinose in the medium. This proof-of-concept experiment assessed the viability of an inducible nitrogen-fixing system in *K. variicola*. The addition of 1.33 mM arabinose to the medium successfully induced nitrogenase activity, and measuring the ammonium excreted in response to different arabinose concentrations not only confirmed efficient ammonium excretion outside the cells but also revealed a titrated response dependent on the inducer concentration.

The team is currently exploring using bacterial biosensors capable of detecting plant metabolites such as flavonoids and phenolic acids found in plant root exudates to replace *nifL* and drive the expression of *nifA*. *Klebsiella* strains were genetically engineered with flavonoid biosensor plasmids to assess their operational range and to determine if cereal root exudates contain sufficient signal molecules to activate them. Ongoing work involves integrating these biosensors into the *Klebsiella* genome to disrupt *nifL* and express *nifA*, thus advancing the development of inducible ammonium-excreting diazotrophs.

Batista, M. B., et al. 2019. "Manipulating Nitrogen Regulation in Diazotrophic Bacteria for Agronomic Benefit," *Biochemical Society Transactions* **47**(2), 603–14.

De Paepe, B., et al. 2018. "Modularization and Response Curve Engineering of a Naringenin-Responsive Transcriptional Biosensor," *ACS Synthetic Biology* **7**(5), 1303–14. DOI:10.1021/acssynbio.7b00419.

Venkataraman, M., et al. 2023. "Synthetic Biology Toolbox for Nitrogen-Fixing Soil Microbes," *ACS Synthetic Biology* **12**(12), 3623–34.

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Achieving High Productivity of 2-Pyrone-4,6-dicarboxylic acid (PDC) from Aqueous Aromatic Streams with *Novosphingobium aromaticivorans*

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Project Goals: Evaluate bioreactor conditions to improve the production of 2-pyrone-4, 6-dicarboxylic acid (PDC) from plant-derived aromatics using *Novosphingobium aromaticivorans.*

Researchers found that the accumulation of intermediate compounds such as protocatechuic acid (PCA) and 3-O-methylgallate (3-MGA) is the major factor for system failure. As such, the team determined that operational conditions that prevented protocatechuic acid (PCA) accumulation during aromatic metabolism improved bioreactor performance. In addition, researchers found that the accumulation of sodium (Na^+) by the addition of sodium hydroxide (NaOH) for maintaining pH-base inhibits the growth and PDC production of *N. aromaticivorans,* and that ammonium (NH_{4+}) has less inhibitory effect than Na^+. PDC productivity increased when using ammonium hydroxide (NH_4OH) instead of NaOH for pH control. Productivity was also increased when the hydraulic retention time in the reactor was reduced to 4 hours. At the best operational condition, a stable PDC production of 1.8 g PDC/L/hr was obtained, which is higher than the highest reported PDC productivity, albeit with a lower product titer of 42 mM (7.9 g/L). Overall, the team's findings demonstrate that the use of a membrane bioreactor with optimizing strategies can significantly enhance the productivity of PDC from plant-derived aromatics. This approach can be applied for production of other valuable chemicals from lignin and additional feedstocks to reduce the selling price of products, thereby contributing to the commercialization of lignocellulose and other renewal materials.

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Charting the Path to Optimize Polysaccharide Accumulation in Bioenergy Sorghum

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http://www.glbrc.org

Project Goals: Engineering plants with polysaccharides that can be easily convertible to bioproducts and specialty biofuels.

Plants leverage sugars for various essential functions, including energy production, building cells and organs, and transmitting signals. Notably, a significant portion of sugars is channeled towards building the cell wall, a predominant component of plant biomass for bioenergy applications. Mixed-linkage glucan (MLG), a vital cell wall constituent in grasses, has emerged as a promising polysaccharide in bioenergy-related applications due to its abundance in easily fermentable glucans and positive impact on plant biomass digestibility. The team's work demonstrated that the abundance of MLG is controlled through development along with cell-type specificity by the action of both MLG synthase and hydrolases. This unique modulation of sugar levels in cells through cell-wall polysaccharides appears distinctive to MLG and sets it apart from other cell-wall polysaccharides in vegetative tissues. Therefore, researchers have been working to improve plant biomass by storing more MLG in the cell wall that can be easily converted into readily available forms of sugar and increase cell wall digestibility.

Due to its favorable attributes as a biofuel feedstock, such as high biomass yield, resilience to adverse environmental conditions, and low resource requirements, researchers focused on sorghum for the manipulation of MLG biosynthesis. The team demonstrated that overexpression of the major MLG synthase, CSLF6, resulted in significant MLG production and accumulation in transgenic sorghum (CSLF6OX), both in the greenhouse and field conditions. However, the team also observed a developmental degradation of MLG in CSLF6OX sorghum. To mitigate this degradation phenomenon, researchers embarked on the manipulation of the MLG degradation pathway. This strategy involved the identification and characterization of MLG hydrolases, also known as lichenases, with a particular focus on the major lichenase enzymes in sorghum. Using bioinformatics tools, researchers identified three sorghum lichenase candidates that possess a signal peptide for cell-wall secretion and a GH17 domain indicative of hydrolase activity as well as high sequence similarity to known lichenases in diverse species. Using synthetic substrates and natural flours, the team optimized the experimental conditions and established the activities of three putative sorghum lichenases. Subsequently, researchers performed a multifaceted approach, including qRT-PCR, RNAseq, *in situ* hybridization, and concurrent MLG and starch quantification throughout different stages of sorghum leaf development in diurnal conditions. The results revealed cell-type-specific, organ-specific, and development-dependent regulation of MLG levels. This regulation is achieved through precise control of CSLF6 and lichenase enzyme levels in different cell types. As a result, the team identified SbLCH1 as the predominant lichenase enzyme in sorghum. Currently, researchers are generating a knockout line of the major SbLCH1 that researchers identified, using CRISPR-Cas9 gene-editing technology. The team's ultimate goal is to integrate a SbLCH1 knockout line with the CSLF6OX hybrid energy sorghum, which researchers have recently developed. By employing this integrated approach, the team anticipates facilitating MLG accumulation by preventing lichenase-dependent degradation, thereby enhancing the accumulation of easily extractable polysaccharides suitable for biofuel production.

Kim, S. J., et al. 2023." Cell- and Development-Specific Degradation Controls the Levels of Mixed-Linkage Glucan in Sorghum Leaves," *Plant Journal* **116**(2), 360–74. DOI:10.1111/tpj.16376.

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Evolutionary Flexibility and Rigidity in the Bacterial Methylerythritol Phosphate Pathway

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Project Goals: Identify potential alternative routes in the bacterial methylerythritol phosphate (MEP) metabolic pathway, which is used to generate high-value terpenoid products.

Terpenoids are a diverse class of compounds with wide-ranging uses such as industrial solvents, fragrances, and more. Industrial production of most terpenoids relies on nonrenewable feedstocks making alternative production methods desirable. Fermentation of engineered microbes using renewable feedstocks like lignocellulose is an attractive strategy for large-scale production of key terpenoids because it has the potential to be sustainable and relatively inexpensive. To achieve large-scale production of terpenoids, there are widespread efforts to engineer the metabolic pathway that generates terpenoids. All terpenoids are made from the final products of the methyl erythritol phosphate (MEP) pathway, which is composed of seven enzymatic steps. Efforts in engineering the MEP pathway have identified some of these enzymes as having unfavorable characteristics, so researchers are interested in identifying alternative enzymatic routes which may have evolved to be functionally redundant to the canonical MEP pathway. The team used comparative genomics to search for alternative enzymes to the canonical MEP enzymes and found that enzymes early in the pathway likely evolved alternatives, as supported by literature. In contrast, enzymes late in the pathway appear to have no alternatives in the database of 4,400 genomes in this study. Early pathway flexibility suggests that researchers may be able to identify the genes responsible for an incomplete canonical pathway and implement alternative enzymes via metabolic engineering should they have more favorable qualities. For the late pathway steps, if alternative enzymes have evolved at all, they are rare or their host organisms have not been sequenced. The evergrowing repository of sequenced bacterial genomes has great potential to provide metabolic engineers with alternative metabolic pathway solutions. The finding that late MEP pathway enzymes are evolutionarily indispensable informs both metabolic engineering efforts and understanding evolving terpenoid biosynthesis pathways.

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The Plant Synthetic Biology Shared Research Objective: Building a Cross-BRC Repository of Regulatory Elements and Testing Technologies

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Plants represent ideal chassis for metabolic engineering and light-driven synthetic biology. Their specific tissues and dedicated organelles give access to unique metabolite pools and allow insulation of newly installed traits. To fully harness untapped potential and enable expression of complex traits, it is critical to develop a portfolio of tools to control gene expression and efficient testing and implementation technologies. The ultimate goal of this shared research objective is a shared standardized repository of validated biobricks, plant synthetic biology parts, and technologies for improving bioenergy feedstocks. Researchers have initiated the cross-BRC repository and are populating it with regulatory elements from sorghum and poplar—tunable promoters that are organ-, tissue-, and cell-type specific and treatment responsive. In this project, researchers showcase identification of genes specifically expressed in cell types of the sorghum stem, epidermis, and root hairs, as well as a highly xylem-specific gene in poplar. Expression analysis revealed complex coexpression networks, including as-yet-uncharacterized genes.

Cloning, validation, and functional characterization of the respective regulatory elements enabled assembly into logic gates and genomic circuits. Tools for functional testing of promoters, delivery of multigene constructs, and rapid

transient expression technology are in development. Application of these tools can enable scalable biosustainable production of natural products and tuning of the plant's capacity for adaptation to and interaction with their biotic and abiotic environment.

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Understanding the Molecular Rules of Transporter Specificity to Engineer Biofuel-Relevant Efflux Pumps

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Project Goals: Researchers used deep mutational scanning to learn the molecular rules of specificity for a multidrug efflux pump. Next, the team will apply these rules to engineer transporters to efflux and confer resistance to toxins found in lignocellulosic hydrolysates, a major barrier to efficient biofuel production.

Transporter engineering offers the ability to precisely control which molecules remain in a cell. This could greatly improve the cost and efficiency of microbial biofuel production, for example by exporting biofuel end products for easier recovery, removing reaction byproducts to prevent toxic buildup, and conferring efflux-mediated resistance to lignocellulosic hydrolysate (LCH) inhibitors. LCH inhibitors can reduce the efficiency and yield of microbial biofuel production and are costly to remove from pretreated plant biomass. Researchers aim to engineer LCH inhibitor efflux pumps using bacterial multidrug resistance (MDR) transporters as a platform. In doing so, researchers will learn design rules for engineering other biofuel-relevant transporters. To achieve this, researchers must first understand the sequence determinants of transport specificity (i.e., how does each residue contribute to efflux of different substrate types?). To this end, researchers have used deep mutational scanning to characterize all single-missense mutants of a bacterial MDR transporter in the context of several toxic molecules, including LCH inhibitors.

Exposure of a variant library to toxic transporter substrates results in enrichment of active variants when analyzed by deep sequencing. Researchers have identified general and substrate-specific functional hotspots and gain-of-function pathways using this method. Next, the team will use this data in combination with machine learning protein design techniques to design LCH inhibitor efflux pumps.

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High (School) Throughput Screening of BAHD Transferases

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Project Goals: BAHD acyltransferases represent a large family of enzymes typically found in plants. They use acyl-CoA donors (produced from acyl-CoA ligases) to form esters or amides with alcohol or amine acceptor molecules. The products of these reactions are incorporated into large polymers (e.g., lignin and suberin) or into small secondary metabolites (e.g., phenolic esters, antimicrobials, antifungals, or compounds that contribute to drought resistance). The goal is to elucidate the identities and functions of these enzymes and use them in conjunction with acyl-CoA ligases to precision-engineer bioenergy crops (Chaudhury et al. 2023). Pairing specific acyl-CoAs and BAHD transferases can allow fine-tuning of lignin content for simple deconstruction (e.g., Zip-Lignin) or by incorporating useful aromatics that can easily be "clipped-off," increasing the net value of the plant biomass.

BAHD acyltransferases can produce valuable molecules in bioenergy crops. The discovery and characterization of a specific BAHD acyltransferase led to the creation of Zip-Lignin technology, in which introduction of ester-linked monolignols allows hydrolysis under mild conditions, avoiding harsher chemical treatments needed to remove lignin during bioenergy processing (Wilkerson et al. 2014). Further investigation showed that specific aromatics could be incorporated into terminal lignin positions, such as *p*-hydroxybenzoate, that can easily be clipped off due to their attachment via an ester linkage (de Vries et al. 2022). Thus, the ability to tune lignin composition not only enables

improved deconstruction but also positions lignin as an attractive source of energy-rich molecules.

By taking advantage of continually improving genomic data and tools, the team curated lists of high-potential target genes focusing on two priority bioenergy crops and a model plant (poplar, sorghum, *Arabidopsis*). Selected genes were synthesized into cell-free expression vectors by the DOE Joint Genome Institute and were then screened using a wheatgerm cell-free system (Cell Free Sciences) by a team of high school–student laboratory members. The expressed proteins were screened for potential activity and categorized by their preferred substrates. Active enzymes catalyzing interesting reactions were then introduced into *Populus* sp. to assess *in vivo* impacts (de Vries et al. 2022; Gonzales-Vigil et al. 2021; Smith et al. 2022), and cell-based expression systems such as *Escherichia coli* have been used to facilitate structural and biochemical characterization. The work presented here has improved understanding of the breadth of molecules this large family of enzymes can synthesize and how these molecules may be useful in producing more energy-efficient plants or providing engineered plant sources for fine specialty chemicals.

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Wilkerson, C. G., et al. 2014. "Monolignol Ferulate Transferase Introduces Chemically Labile Linkages into the Lignin Backbone," *Science* **344**, 90–93. DOI:10.1126/science.1250161.

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Bioenergy Research Centers
Joint BioEnergy Institute (JBEI)

Mapping Enzymatic Esterification to Natural Expression Levels for a Specialized Clade of HCT Acyltransferases in Poplar

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Project Goals: Hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl transferases (HCTs) are an important group of BAHD acyltransferase enzymes because of their key roles in the lignin biosynthetic pathway and the biosynthesis of plant specialized metabolites, such as chlorogenic acid (caffeoylquinic acid) and *p*-coumaroyl shikimate. The inherent promiscuity of the BAHD acyltransferases leads to a combinatorial array of plant natural products and offers interesting targets for both enzyme and plant engineering. Many plants have large HCT families, but the differences in substrate specificity are not well understood.

To address this knowledge gap, researchers screened all *Populus trichocarpa* BAHD acyltransferases for HCT activity and found a distinct clade of nine enzymes with various activities. Results show two distinct catalytic classes of HCTs in poplar residing in different branches of the clade: shikimate-specific enzymes (HSTs), likely involved in lignin biosynthesis based on expression data, and quinate-preferring enzymes (HQTs). The extent of substrate promiscuity and competitive preferences of the different enzymes were also determined. Both the HST and HQT enzymes were found to generate shikimate or quinate ester products and convert the products back into CoA thioester and acid substrates under appropriate reaction conditions. Active site residues potentially involved in switching the reaction specificity between HSTs and HQTs were identified through AlphaFold protein structure analysis and tested for their roles in defining HCT substrate specificity and activity by site-directed mutagenesis. Insights from this work will be presented.

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Performance Thresholds for Co-Utilization of Lignin-Derived Aromatics and Sugars

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Project Goals: Enhance understanding of the economic viability of noncombustion lignin utilization routes and determine the minimum conversion threshold from lignin stream to product that must be achieved.

Efficient lignin conversion is vital to the production of affordable, low-carbon fuels and chemicals from lignocellulosic biomass. However, lignin conversion remains challenging, and the alternative (i.e., combustion) can emit harmful air pollutants. This study explores the economic and environmental tradeoffs between lignin combustion and microbial utilization for producing bisabolene as a representative biobased fuel or chemical. Considering three different biomass feedstocks—biomass sorghum, switchgrass, and clean pine—the project primarily addressed two important questions: (1) what quantity of lignin must be utilized by the host microbe to render the strategy of co-utilizing sugars and lignin-derived bioavailable intermediates economically feasible and (2) what proportion of lignin can be utilized while still achieving the Renewable Fuel Standard life-cycle greenhouse gas (GHG) emissions goal of a 60% reduction relative to petroleum equivalent.

Results for switchgrass and clean pine–based biorefineries show that using lignin to increase fuel yields rather than

* Presenting Author

combusting it reduces the capital expenditures for the boiler and turbogenerator if the facilities process more than 1,100 bone-dry tons (bdt) of feedstock per day and 560 bdt/day, respectively (Baral et al. 2023). No comparable advantage was observed for lower-lignin sorghum feedstock. Deconstructing lignin to bioavailable intermediates and utilizing those small molecules alongside sugars to boost product yields is economically attractive if the overall lignin-to-product conversion yield exceeds 11 to 20% by mass (Baral et al. 2023). Although lignin-to-fuel/chemical conversion can increase GHG emissions, most of the lignin can be diverted to fuel/chemical production while maintaining a >60% life-cycle GHG footprint reduction relative to diesel fuel (Baral et al. 2023). The results underscore that lignin utilization can be economically advantageous relative to combustion for higher-lignin feedstocks, but efficient depolymerization and high yields during conversion are both crucial to achieving viability.

Baral, N. R., et al. 2023. "Economic and Environmental Trade-Offs of Simultaneous Sugar and Lignin Utilization for Biobased Fuels and Chemicals," *ACS Sustainable Chemistry & Engineering* **12**(7), 2563–76.

Automation of a CRISPRi Platform for Enhanced Isoprenol Production in *Pseudomonas putida*

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https://www.jbei.org

Project Goal: Establish scientific knowledge and new technologies to transform the maximum amount of carbon available in bioenergy crops into biofuels and bioproducts.

Automation technologies expedite design-build-test-learn (DBTL) cycles while reducing time and resources. Here, the project developed an automated conversion pipeline that, coupled with machine learning, omics studies, and microfluidics, elevates the capacity to engineer microbes. *Pseudomonas putida* is a promising microbial host owing to its genetic tractability and capacity to grow on many carbon substrates (Wang et al. 2022). Recently, *P. putida* was engineered for isoprenoid production with subsequent work improving isoprenol titer. Isoprenol is a biological precursor to 1,4-dimethylcyclooctane (DMCO), a sustainable aviation fuel (Baral et al. 2021). Building upon that work, researchers applied CRISPR interference (CRISPRi), which uses a deactivated Cas9 enzyme (dCas9) and customizable guide RNAs (gRNAs) to selectively downregulate orthogonal metabolic pathways. The team developed an automated pipeline to rapidly screen CRISPRi targets and test strains for isoprenol production. The pipeline harnessed microfluidic liquid handlers (ECHO, Mantis, and Biomek) for nanoliter-scale dispensing of molecular cloning reagents, then employed a customized high-throughput electroporation device for rapid transformation of 384 strains in parallel. Growth and production studies were completed in BioLector microfermentors with proteomics data collected to verify dCas9 expression and confirm gene of interest downregulation. Finally, researchers trained a machine learning model, the Automated Recommendation Tool (ART), with isoprenol titers and associated downregulated genes to iteratively generate recommendations for further downregulation (Radivojević et al. 2020). When coupled with automation, CRISPRi enabled researchers to screen 130 genes associated with isoprenoid precursors or utilization pathways in parallel to identify genes that improve isoprenol titer. Using ART, genes were then iteratively paired in 2- and 3-gRNA arrays for further investigation. CRISPRi successfully downregulated selected genes to increase metabolic flux towards isoprenol in *P. putida* and, by iteratively screening different target combinations in gRNA arrays, demonstrably improved titers. Following this successful application, the team plans to use ART to further explore and exploit gRNA combinations to maximize isoprenol titer. The pipeline demonstrates a successful application of machine learning tools to systematically and predictably improve isoprenol titers, rates, and yields.

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Wang, X., et al. 2022. "Engineering Isoprenoids Production in Metabolically Versatile Microbial Host *Pseudomonas putida*," *Biotechnology for Biofuels and Bioproducts* **15**(1), 137.

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Modulating Bioenergy Traits in Field-Grown Sorghum Affects the Rhizosphere Bacterial Communities

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Project Goals: Determine how bioenergy crop engineering affects interactions with soil microbes.

Engineering bioenergy crops for reduced cell wall recalcitrance and increased production of high-value chemicals represents a promising approach to sustainably produce biofuels from both economic and environmental standpoints. Yet, the impact of modifying bioenergy traits in feedstocks on the indigenous soil microbiome of agricultural lands remains largely uncharacterized. The project profiled the rhizosphere bacterial communities associated with transgenic sorghum lines engineered for reduced cell wall recalcitrance (AT10) and increased protocatechuate production (Qsub) via 16S amplicon sequencing. The team found that the rhizosphere bacterial community composition was different between the transgenic sorghum lines and wildtype. In the rhizosphere of transgenic lines, researchers detected an enrichment of the phyla (Actinobacteriota, Bacteroidota, and Proteobacteria) that are generally considered copiotrophs, which thrive on labile carbon and grow rapidly under nutrient-rich conditions. Conversely, the team observed a depletion in the abundance of oligotrophic phyla (Planctomycetota, Acidobacteriota) known for adapting to nutrient-poor conditions and having a slower growth rate. These findings suggest that reducing cell wall integrity and increasing secretion of bioproducts may favor and induce the proliferation of copiotrophic bacteria in soil. Such alterations in soil microbial communities could impact soil health and various soil processes, including nutrient turnover and greenhouse gas emissions, highlighting the importance of further investigation.

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Engineering Poplar for Production of Coproducts Muconic Acid and 2-Pyrone-4,6-Dicarboxylic Acid

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Project Goals: Designing and testing metabolic engineering strategies for production of the value-added coproducts muconic acid (MA) and 2-pyrone-4,6-dicarboxylic acid (PDC) in woody biomass of the bioenergy crop poplar.

1. Identifying key enzymes that can be manipulated to divert metabolic flux towards the production of MA and PDC.
2. Establishing expression cassettes with stacking of multiple genes involved in MA or PDC biosynthesis and transforming them into poplar.
3. Analyzing transgenic plants for production of the desired MA and PDC.
4. Observing the effects of pathway engineering on plant growth and development, and woody biomass processability.

Increasing fossil fuel consumption and concerns about climate change and energy security have been driving the worldwide revolution towards more sustainable and renewable energy sources derived from biomass, agricultural crops, and waste materials. However, the high processing cost of biofuel production hampers its application. Production of high-value coproducts in biomass has been regarded as a solution to reduce the overall cost of biofuel biorefining (Yang et al. 2020). In this project, researchers tested the possibility of redirecting the shikimate pathway metabolic flux towards the production of valuable chemicals MA and PDC through systematic metabolic engineering in the bioenergy crop poplar. MA serves as a platform chemical for the manufacture of functional resins, bioplastics, food additives, agrochemicals, and pharmaceuticals, and for generation of bulk chemicals like adipic acid, terephthalic acid, and trimellitic acid (Eudes et al. 2018). PDC is used for manufacturing performance-advantaged biodegradable polymers with strong adhesive properties, high elasticity, and rigidity (Lin et al. 2021). For MA production, the salicylic acid pool derived from chorismate via the shikimate pathway is converted to catechol and MA by the sequential activities of bacterial salicylate hydroxylase (NahG) and catechol 1, 2-dioxygenase (CatA). Multiple genes (*AroG**, *Irp9, NahG,* and *CatA*), driven by the xylem preferential

expression promoters, were stacked and transformed into *Populus tremula* x *P. alba* via agrobacterium-mediated gene transformation. Transgenic lines grew similar to the vector control plants and did not show detrimental growth defects. LC-MS analysis of stem samples showed a 3x higher accumulation level of catechol in transgenic lines than in the control plants, and up to 9 µg/g dry weight MA production. For PDC production, five genes (*AroG*, *QsuB*, *PmdA*, *PmdB*, and *PmdC)* were stacked on an expression cassette directed into plastid and transformed in hybrid poplar. The transgenic plants exhibited strong growth defects compared to the wildtype plants under normal conditions. LC-MS analysis of soluble phenolics in the leaves of transgenic plants revealed a substantial increase in the accumulation level of protocatechuate (PCA), an intermediate for production of PDC, showing 40 to 100 times higher levels compared to the wildtype plants. These results demonstrate the potential of engineering the production of value-added chemicals in poplar, highlighting the importance of genetic manipulation and optimization of metabolic pathways for biomass-based biorefinery applications. These findings also pave the way for further research and development in utilizing plant-based production of sustainable and renewable alternatives to petroleum-derived chemicals.

Eudes, A., et al. 2018. "Production of Muconic Acid in Plants," *Metabolic Engineering* **46**, 13–19.

Lin, A., 2021. "*In-Planta* Production of the Biodegradable Polyester Precursor 2-Pyrone-4,6-Dicarboxylic Acid (PDC): Stacking Reduced Biomass Recalcitrance with Value-Added Co-Product," *Metabolic Engineering* **66**, 148–56.

Yang, M., et al. 2020. "Accumulation of High-Value Bioproducts in Planta Can Improve the Economics of Advanced Biofuels," *The Proceedings of the National Academy of Sciences* **117**(15) 8639–48.

Funding Statement: This work was supported by the Joint BioEnergy Institute, one of the Bioenergy Research Centers of the DOE Office of Science, BER program through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and DOE.

EcoFAB 3.0: A Controlled Ecosystem for Bioenergy Crops

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https://www.jbei.org/

Project Goals: The Joint BioEnergy Institute's mission is to establish the scientific knowledge and new technologies in feedstock development, deconstruction and separation, and conversion needed to transform the maximum amount of carbon available in bioenergy crops into biofuels and bioproducts.

Plant–microbe interactions are critical to sustainable agriculture. Technologies are needed that enable studying these interactions under sterile and controlled laboratory conditions to decouple their complexities and identify molecular mechanisms. Several existing systems include EcoFABs, RootArray, RootChip, and Tracking Root Interaction System (TRIS). Typically, each system has advantages for studying different aspects of plant–microbe interactions. Researchers have demonstrated the repeatability of observations using these platforms. For example, EcoFAB 2.0 was recently used to study the effects of nitrogen starvation on root exudates (Novak et al. 2024). Stanley et al. (2018) used RootChip to observe asymmetric root hair growth in response to an asymmetric phosphate perfusion. However, these platforms are designed for small model plants such as *Brachypodium distachyon* and *Arabidopsis thaliana.* This leaves a gap in translating findings from model plants to economically significant bioenergy crops such as sorghum.

In this work, researchers introduce a novel platform (EcoFAB 3.0) designed to study bioenergy plants such as sorghum for up to 4 weeks in a sterile and controlled environment. In addition to its larger size, EcoFAB 3.0 addresses many other limitations of previously used platforms. Its 2-step assembly makes it easy to set up and is highly user-friendly. Its root chamber is dark and allows the roots to grow more naturally by using a rhizotron-like window for imaging. The device has several multipurpose ports which can be used for exudate collection, ventilation, and introducing various sensors for monitoring gaseous exchange, temperature, moisture, and other parameters. Excitingly, researchers have now found that EcoFAB 3.0 is able to recreate field and greenhouse observations made by Lin et al. (2022), showing that an engineered line of sorghum has higher accumulation of 4-hydroxybenzoic acid and lower biomass in comparison to wildtype.

Lin, C.-Y., et al. 2022. "Engineering Sorghum for Higher 4-Hydroxybenzoic Acid Content," *Metabolic Engineering Communications* **15**, e00207.

Novak, V., et al. 2024. "Reproducible Growth of Brachypodium in EcoFAB 2.0 Reveals that Nitrogen Form and Starvation Modulate Root Exudation," *Science Advances* **10**, eadg7888.

Stanley, C. E., et al. 2018. "Dual-Flow-RootChip Reveals Local Adaptations of Roots Towards Environmental Asymmetry at the Physiological and Genetic Levels," *New Phytologist* **217**, 1357–69.

Funding Statement: This work was part of the DOE Joint BioEnergy Institute (http:// www.jbei.org) supported by the DOE Office of Science, BER program through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and DOE.

Conversion of Natural and Transgenic Sugarcane and Poplar Variants with an Ionic-Liquid-Based Feedstocks-To-Fuels Pipeline

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The four DOE Bioenergy Research Centers (BRCs) have recently launched extensive formal collaborative projects with the goal of accelerating the common science goals for the bioenergy enterprise. Each BRC has integrated process concepts and feedstocks that feature novel deconstruction technologies and conversion platforms that transform plants to biofuels and bioproducts. In particular, the Joint BioEnergy Institute has developed a Feedstocks-to-Fuels pipeline for screening the efficiency of deconstruction and microbial conversion of lignocellulosic biomass. Here, researchers present results obtained from subjecting two types of bioenergy feedstocks (sugarcane and poplar) to this pipeline consisting of an ionic liquid pretreatment process, enzymatic saccharification, and microbial conversion to the jet-fuel precursor bisabolene.

In this study, researchers explore the conversion potentials of two engineered sugarcane varieties that produce high levels of lipids (oilcane 1565 and 1566) and the CP88 wildtype (WT), developed at the Center for Advanced Bioenergy and Bioproducts Innovation. The starting material for the conversion experiments was the bagasse obtained after pressing, drying, and milling the biomass. Researchers also evaluated the use of natural poplar variants with different wood density developed at the Center for Bioenergy Innovation. Poplar biomass with higher wood density can increase biomass yield per hectare while reducing transportation costs, but the impact of density on the degree of conversion to sugars and bioproducts remains unexplored. Pretreatment was performed using the ionic liquids cholinium lysinate and ethanolamine acetate at different concentrations (10 to 85 wt%), with 15 wt% solids loading in high-pressure and high-temperature glass tube reactors at 140 °C for 3 hours. The pretreated feedstocks underwent enzymatic hydrolysis using a cellulase:hemicellulase enzyme cocktail (9:1 vol/vol, 30 mg/g biomass) and incubated at 50 °C for 72 hours. Compositional analysis of the raw and pretreated samples was performed using a 2-step acid hydrolysis method and high-performance liquid chromatography was used to quantify the sugars released during this reaction and after enzymatic hydrolysis. The WT sugarcane contained 37% glucan, while engineered sugarcane contained 39%, with lignin content below 24% for both WT and engineered sugarcane. Pretreatment reduced lignin by 1.5-fold and increased glucan 1.3-fold. Ethanolamine acetate performed better than cholinium lysinate with engineered sugarcane, yielding 88% glucose and 57% xylose, while both solvents achieved over 94% glucose and 70% xylose yield for WT. Fermentation with *Rhodosporidium toruloides* showed near-complete glucose consumption and bisabolene production, underlining the effectiveness of the pipeline for conversion of different bioenergy feedstocks to bioproducts.

Understanding the Role of Tolerance on Microbial Production of Isoprenol

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https://www.jbei.org/

Project Goals: Discovery, optimization, and enhancement of tolerance mechanisms in bacterial hosts to biomass-related inhibitors and final products to generate robust, scalable production platforms.

Isoprenol (3-methyl-3-buten-1-ol), a precursor for diverse commodity chemicals (Baral et al. 2021), can be converted

into 1,4-dimethylcyclooctane (DMCO), which can further be used as a sustainable aviation fuel blendstock (Baral et al. 2021). Bioproduction of isoprenol has been reported from various engineered bacterial hosts such as *Escherichia coli*, *Corynebacterium glutamicum*, and *Pseudomonas putida* (Kang et al. 2016; Sasaki et al. 2019; Wang et al. 2022; Banerjee et al. 2024). A critical factor affecting scalability for high titer rate–yield production of isoprenol in these microbial hosts is its toxicity. Therefore, it is imperative to understand and improve the tolerance to isoprenol in these bacterial hosts that affect both growth and production.

In the current study, this team specifically focused on *P. putida* KT2440, an ideal production chassis due to its fast growth rate, capability to utilize various substrates including lignin aromatics, and high stress tolerance, which are critical factors in industrial bioprocesses (Nikel and de Lorenzo 2018). *P. putida* has endogenous isoprenol catabolism pathways and, hence, the native regulatory cascades driving the microbial physiology could be perturbed upon heterologous production of isoprenol. The group used a top-down approach harnessing the power of adaptive laboratory evolution to generate isoprenol-tolerant phenotypes and further characterize them (Lim et al. 2021). For this purpose, the team tolerized wildtype, as well as mutant strains of *P. putida* lacking isoprenol catabolism, in glucose minimal medium by gradually increasing the concentration of isoprenol.

The group successfully obtained evolved strains that could robustly grow in the presence of 8 grams per liter isoprenol compared to the basal strain that was unable to grow at this concentration. Furthermore, the team utilized whole-genome sequencing, gene expression profiling (RNA sequencing), and global proteomics to understand determinants of isoprenol tolerance in these novel, evolved isolates compared to the parent strains. Researchers also study how these profiles change when they heterologously express isoprenol production pathways in these evolved isolates and the subsequent effects on production. Taken together, unraveling and understanding the effects of the evolution-driven isoprenol tolerization mechanism and its effects on production in this important bacterial chassis will help scientists rationally engineer robust isoprenol production platforms.

Banerjee, D., et al. 2024. "Genome-scale and Pathway Engineering for the Sustainable Aviation Fuel Precursor Isoprenol Production in *Pseudomonas putida*," *Metabolic Engineering* **82**, 157–70. DOI:10.1016/j.ymben.2024.02.004.

Baral, N. R., et al. 2021. "Production Cost and Carbon Footprint of Biomass-Derived Dimethylcyclooctane as a High Performance Jet Fuel Blendstock," *ACS Sustainable Chemistry & Engineering* **9**(35),11872–82. DOI:10.1021/acssuschemeng.1c03772.

Kang, A., et al. 2016. "Isopentenyl Diphosphate (IPP)-Bypass Mevalonate Pathways for Isopentenol Production," *Metabolic Engineering* **34**, 25–35. DOI:10.1016/j.ymben.2015.12.002.

Lim, H. G., et al. 2021. "Generation of *Pseudomonas putida* KT2440 Strains with Efficient Utilization of Xylose and Galactose via Adaptive Laboratory Evolution," *ACS Sustainable Chemistry & Engineering* **9**(34),11512–23. DOI:10.1021/acssuschemeng.1c03765.

Nikel, P. I., and V. de Lorenzo. 2018. *Pseudomonas putida* as a Functional Chassis for Industrial Biocatalysis: From Native Biochemistry to Trans-Metabolism. *Metabolic Engineering* **50**,142–55. DOI:10.1016/j.ymben.2018.05.005.

Sasaki, Y., et al. 2019. "Engineering *Corynebacterium glutamicum* to Produce the Biogasoline Isopentenol from Plant biomass Hydrolysates," *Biotechnology for Biofuels* **12**(41). DOI:10.1186/s13068-019-1381-3.

Wang, X., et al. 2022. "Engineering Isoprenoids Production in Metabolically Versatile Microbial Host *Pseudomonas putida*," *Biotechnology for Biofuels and Bioproducts* **15**(1). DOI: 10.1186/s13068-022-02235-6.

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Energy Earthshots

Root Biosynthesis Engineering of the "Plant Diamond" Sporopollenin for Permanent Belowground Carbon Storage

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Project Goal: The goals of this project are to (1) identify the genes required for sporopollenin synthesis and deposition and (2) introduce their expression in *Populus* to engineer sporopollenin production in roots as a stable carbon (C) sink.

This project is part of the DOE Carbon Negative Shot™ program, which calls for research on atmospheric carbon dioxide (CO_2) removal and storage. To increase C storage, researchers propose to engineer the production of sporopollenin—the most recalcitrant plant polymer known (biostable for centuries or more versus decades for other biopolymers)—in roots of bioenergy crops. Researchers will target *Populus* species since these trees are among DOE's most important crops to be used for bioenergy.

In the first part of this project, the team will attempt to synthesize sporopollenin in *Populus* root epidermal cells, based on current knowledge of sporopollenin synthesis genes and epidermis-specific regulation of gene expression. More specifically, researchers will induce the expression of the presently known core set of enzymes needed to synthesize sporopollenin precursors—the *Populus* orthologs of ACOS5, PKSA, PKSB, TKPR1, and MS2 and the proposed master transcription factor regulators of sporopollenin synthesis AMS and MS188. Gene constructs driven by a root epidermis–specific promoter will be introduced into *Populus* via hairy root transformation of *in vitro*–grown shoots with *Agrobacterium rhizogenes*.

The second component of this project will use single-cell genomics to uncover genes that are activated during tapetum development and sporopollenin synthesis in *Arabidopsis* and *Populus*. Researchers' aim is to identify regulatory genes involved in this process that have not yet been characterized and define evolutionarily conserved genetic mechanisms of sporopollenin synthesis, which is likely to be essential for the transfer of this cellular role to other cell types or species. The team will validate the function of these genes in *Arabidopsis* before adding them to the *Populus* genetic toolkit developed in the first part of this project.

The proposed strategy, deployed at scale, has the potential to strip substantial amounts of C from the atmosphere. Based on typical *Populus* biomass yields and allocation belowground, engineering roots to contain 5% by weight sporopollenin could permanently store 120 to 300 kg CO_2-equivalents per hectare per year or 96 to 2,400 kg per hectare in an 8-year cycle. Researchers estimate that engineering the 36-million-hectare U.S. maize crop to accumulate 5% sporopollenin in roots and stover could sequester ~50 megatons of CO_2-equivalents per year, or ~0.5 gigatons per decade.

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Unleashing Photosynthesis and Nitrogen Fixation for Carbon Neutral Production of Nitrogen-Rich Compounds

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Project Goals: Address basic research challenges in developing and advancing technologies for green fertilizer production.

Nitrogen is essential for life on Earth. Today, most of life's nitrogen need is met by chemical conversion of atmospheric nitrogen into readily usable forms, but such conversion comes at a massive environmental cost. It is conducted under high temperature and pressure, generating a massive carbon footprint. An alternative approach is based on biological conversion of nitrogen at ambient temperature, a greener process restricted to only a few select groups of microbes. Of these, cyanobacteria are uniquely capable of driving the energetically expensive nitrogen fixation reaction solely with solar power while simultaneously capturing carbon, and thus reducing the carbon footprint (Liberton et al. 2019; Bandyopadhyay et al. 2021).

However, the use of cyanobacteria, nonmodel microbes, as chassis for the conversion of atmospheric nitrogen into valuable nitrogen-rich compounds requires significant fundamental research, including development of robust growth conditions and systems-level understanding of the biology of these photosynthetic autotrophs. This project addresses foundational research challenges that stand in the way of developing nitrogen-fixing cyanobacteria as cell factories for the production of nitrogen-rich compounds. This group focuses on the production of guanidine, ammonia, and urea, three nitrogen-rich compounds that can serve as substitutes for synthetic fertilizers (Wang et al. 2019). Specifically, researchers are (1) designing and building functional modules for the production of guanidine, urea, and ammonia; (2) designing and building nitrogen-fixing chassis strains for optimal carbon and nitrogen capture and product formation; and (3) optimizing the production chassis.

This team is using two strains representing the two contrasting paradigms that cyanobacteria use to accommodate the mutually antagonistic processes of oxygenic photosynthesis and nitrogen fixation: temporal separation in a unicell (*Cyanothece* 51142) and spatial separation in a multicellular filament (*Anabaena* 33047). Researchers are working to engineer novel enzymes capable of catalyzing the conversion of atmospheric nitrogen into guanidine, ammonia, and urea, and membrane transporters that will secrete the products out of the cell. Multiomics studies and machine learning tools will unravel the fundamental principles underlying the regulation of carbon and nitrogen fixation in cyanobacteria and their channelization towards the products of interest. The research team's goal is to develop chassis strains that can produce sufficient quantities of fertilizer compounds for pilot scale-up geared towards commercialization of the concept. In the longer term, this group envisions future deployment of such strains in soil as a local source of nitrogen for bioenergy and other crops, and also in ocean fertilization for carbon dioxide removal. This technology, when fully developed, has the potential to replace the use of synthetic fertilizers. The fundamental knowledgebase this research generates will also have broader scientific impact on carbon neutral biomanufacturing of nitrogen-containing petrochemical replacement compounds.

This research team of seven investigators from Washington University, National Renewable Energy Laboratory, and Alabama State University brings together significant interdisciplinary expertise in cyanobacterial systems biology, metabolic modeling, machine learning, and synthetic biology. An important mission of this project is to train a number of students from underprivileged communities, equipping a future workforce with modern biomanufacturing technologies.

Bandyopadhyay, A., et al. 2021. "Antenna Modification Leads to Enhanced Nitrogenase Activity in a High Light-Tolerant Cyanobacterium," *mBio* **12**(6). DOI:10.1128/mbio.03408-21.

Liberton, M., et al. 2019. "Enhanced Nitrogen Fixation in a *glgX*-Deficient Strain of *Cyanothece* sp. Strain ATCC 51142, a Unicellular Nitrogen-Fixing Cyanobacterium," *Applied and Environmental Microbiology* **85**(7). DOI:10.1128/AEM.02887-18.

Wang, B., et al. 2019. "Photosynthetic Production of the Nitrogen-Rich Compound Guanidine," *Green Chemistry* **21**(11), 2928–37. DOI:10.1039/C9GC01003C.

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University Projects

Enhancing Biological Nitrogen Fixation in Sorghum (*Sorghum bicolor*) Aerial Roots Through Engineering Diazotrophic Communities

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https://sonar.bact.wisc.edu/

Project Goals: Researchers aim to reduce the dependency of bioenergy production on synthetic nitrogen fertilizers by taking better advantage of biological nitrogen fixation. This group specifically focuses on nitrogen fixation in the mucilage produced by aerial sorghum roots (*Sorghum bicolor*). Alongside collaborators exploring this plant trait, the group investigates the sorghum-associated bacterial communities that contribute to biological nitrogen fixation. The plan includes (1) isolating and characterizing bacterial strains, including diazotrophs, from aerial root mucilage; (2) assessing bacterial interspecies interactions that influence biological nitrogen fixation; and (3) developing and testing synthetic communities with robust biological nitrogen fixation capabilities.

Sorghum (*S. bicolor*) is a promising bioenergy crop due to its high biomass yield, resilience to harsh environmental conditions, and ability to grow in diverse geographical regions. However, sorghum production relies on synthetic nitrogen fertilizers, which have negative economic and ecological consequences such as leaching and greenhouse gas production. Group members identified sorghum accessions harboring high rates of biological nitrogen fixation in a carbohydrate-rich gel/mucilage produced by aerial roots. This study used acetylene reduction assays and transfer of this fixed nitrogen to the plant using nitrogen (^{15}N) gas enrichments to demonstrate that high rates of nitrogenase activity occur in the mucilage. Researchers also determined that these sorghum accessions obtain up to 43% of their nitrogen from the atmosphere using ^{15}N isotope dilution experiments (Venado et al. 2023). In this part of the project, the group aimed to (1) isolate bacteria, including diazotrophs, from the sorghum mucilage; (2) explore bacteria interspecies interactions using synthetic communities (SynComs); and (3) explore the potential of engineering diazotrophs from the mucilage to further enhance nitrogen fixation (Chakraborty et al. 2023; Venkataraman et al. 2023).

1. The group isolated over 200 unique bacteria from sorghum mucilage. They assessed carbon utilization, plant growth-promoting activities, and nitrogenase activity of 34 promising diazotrophs along with *Azospirillum brasilense* FP2 and *Klebsiella variicola* A3 as reference strains. Among the 34 new strains tested, at least 23 showed robust nitrogenase activity, with 11 strains showing higher nitrogenase activity than the two reference strains. Pairwise coculture assays indicated that several strains, including non-diazotrophs, enhanced the nitrogenase activity of *A. brasilense* and *K. variicola*, indicating that sorghum mucilage harbors "helper strains" in addition to diazotrophs.
2. The group used a SynCom approach to investigate the bacterial interspecies interactions impacting biological nitrogen fixation. Researchers assessed community nitrogenase activity and time-resolved composition from 93 different subcommunities of PComm1, leading to a dataset containing a range of diazotroph growth and fixation profiles. The group found that nitrogenase activity is positively correlated with diazotroph abundance. However, there were several notable exceptions, suggesting the presence of interspecies competition impacting community fixation that is not growth-mediated. Researchers observed pairwise cocultures containing one diazotrophic and one non-diazotrophic species can lead to improved nitrogenase activity compared to the monoculture, indicating again the presence of strains that can function as helpers for diazotrophs in a species-specific manner. The group is further exploring this dataset using a paired modeling approach.
3. The group genetically engineered *Klebsiella* strains isolated from the mucilage to fix more nitrogen and release this fixed nitrogen as ammonium. Using biosensors, some of these ammonium-excreting diazotrophs were further engineered to release nitrogen only in the presence of arabinose, an abundant sugar in the sorghum mucilage, to improve the fitness of the engineered strains. Additional biosensors are currently being investigated.

Altogether, this project allowed researchers to identify efficient diazotrophs from the sorghum mucilage, better understand interactions between bacteria within this unique

* Presenting Author

environment, and engineer these bacteria to further increase nitrogen fixation rates and the delivery of fixed nitrogen to sorghum, and to enhance the efficiency and sustainability of bioenergy production.

Chakraborty, S., et al. 2023. "Scripting a New Dialogue between Diazotrophs and Crops," *Trends in Microbiology*. DOI:10.1016/j.tim.2023.08.007.

Venado, R. E., et al. 2023. "Mucilage Produced by Sorghum (*Sorghum Bicolor*) Aerial Roots Supports a Nitrogen-Fixing Community," *bioRxiv*. DOI:10.1101/2023.08.05.552127.

Venkataraman, M., et al. 2023. "Synthetic Biology Toolbox for Nitrogen-Fixing Soil Microbes," *ACS Synthetic Biology* **12**(12): 3623–34. DOI:10.1021/acssynbio.3c00414.

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Identification of the Genetic Factors that Contribute to Biological Nitrogen Fixation in Sorghum (*Sorghum bicolor*)

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https://sonar.bact.wisc.edu/

Project Goals: The primary aim of this proposal is to deepen comprehension of the molecular and cellular mechanisms governing associative nitrogen fixation traits within the mucilage of sorghum plants that form aerial roots. This will be achieved through a multifaceted approach encompassing genetics, synthetic bacterial communities, and systems biology. The overarching hypotheses posit that both plant and bacterial gene networks play pivotal roles in regulating nitrogen fixation efficiency in sorghum, and unraveling these networks will facilitate the enhancement of nitrogen fixation in sorghum, thus advancing its potential for bioenergy production.

Current agricultural practices rely heavily on applying nitrogen-rich fertilizers, posing significant environmental risks, including pollution of ground and surface water, nitrous oxide emissions, and greenhouse gas emissions during the production of ammonia-based fertilizer. However, adopting biological nitrogen fixation presents a promising avenue for mitigating these risks. Sorghum (*Sorghum bicolor* L. Moench) is gaining recognition as a sustainable crop due to its resilience to drought and high temperatures, and certain types of sorghum produce high yields of lignocellulosic biomass that can be used to produce renewable fuels and chemicals. Select sorghum accessions exhibit prolific growth of thick aerial roots that secrete a dense, carbohydrate-rich mucilage following rainfall and in humid conditions (Venado et al. 2023). This mucilage is an optimal environment for diazotrophic microorganisms that provide the plant with ammonium. To improve sorghum's ability to support diazotrophs, breeding programs require a detailed understanding of the genetic factors influencing aerial root development and mucilage production. In pursuit of this objective, researchers conducted a comprehensive genome-wide association study (GWAS) on the sorghum minicore that consists of 242 landraces and 30 accessions from the sorghum association panel at two locations (Florida and Wisconsin) and with a standard and reduced fertilizer treatment at each location. Through this GWAS, group members identified loci associated with the phenotypes of aerial root diameter and the number of nodes with aerial roots. Sequence variations within genes responsible for encoding transcription factors governing phytohormone signaling and root system architecture were associated with these traits (Wolf et al. 2023). In parallel, several breeding populations were developed from crosses between accessions that produce aerial roots and regionally adapted sweet sorghums. Segregation of F2 populations was used to validate some of the loci identified in the GWAS, whereas continued inbreeding and selection are expected to result in bioenergy sorghum cultivars capable of obtaining a portion of their nitrogen needs from biological nitrogen fixation. Furthermore, the group conducted single-cell RNA sequencing (scRNAseq) on sorghum aerial roots, comparing those with and without mucilage. This analysis uncovered novel gene markers specific to different cell types. Leveraging the scRNAseq data, group members constructed gene regulatory networks using various algorithms, including single-cell Multi-Task Network Inference (scMTNI). This approach will enable researchers to explore genes essential for mucilage production further. Together, these results offer opportunities for enhancing biological nitrogen fixation in cereal crops and reducing reliance on synthetic fertilizers.

Wolf, E. S. A., et al. 2023. "Identification of Genetic and Environmental Factors Influencing Aerial Root Traits That Support Biological Nitrogen Fixation in Sorghum," *G3:Genes, Genomes, Genetics* **14**(3). DOI:10.1093/g3journal/jkad285.

Venado, R. E., et al. 2023. "Mucilage Produced by Sorghum (*Sorghum Bicolor*) Aerial Roots Supports a Nitrogen-Fixing Community," *bioRxiv*. DOI:10.1101/2023.08.05.552127.

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Investigating Cellular Network and Outer-Membrane Vesicles for the Metabolism of Lignin-Derived Aromatics in Soil *Pseudomonas* Species

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Project Goals: The overall goal of this project is to elucidate the relationship between the cellular metabolic network and the metabolic reactions in outer membrane vesicles secreted by soil *Pseudomonas* species. In particular, the research team aims to evaluate the catabolism of lignin-derived aromatics in *Pseudomonas* strains toward maximizing aromatic catabolic activity via engineered or synthetic cellular and vesicle systems. The results from this work will enhance understanding of carbon cycling by soil bacteria and have implications in the use of engineered pseudomonads for lignin valorization to value-added compounds to support the bioeconomy.

Valorization of lignin is an important component of a sustainable bioeconomy. Soil *Pseudomonas* strains, which natively catabolize lignin-derived aromatics (LDAs), are commonly engineered for the conversion of LDAs to value-added compounds. It was shown that *Pseudomonas putida* secretes outer membrane vesicles (OMVs) enriched with enzymes that catalyze LDA turnover (Salvachúa et al. 2020). However, the metabolic reaction networks of pseudomonad OMVs and their relationships to intracellular metabolism remain uncharacterized.

To reveal how OMVs potentially facilitate *P. putida* utilizing LDAs, potential bottlenecks of cells catabolizing different LDAs were identified by measuring the intracellular metabolite levels in cells fed with ferulate (FER), *p*-coumarate (COU), vanillate (VAN), or 4-hydroxybenzoate (4HB) as the sole carbon source. When *P. putida* was fed with FER and COU, intermediates accumulated in the peripheral pathways involved in the conversion of FER to VAN and COU to 4HB, suggesting the presence of bottlenecks in these pathways. Specifically, in FER-fed *P. putida*, vanillin was 20-fold higher than its upstream metabolite feruloyl-CoA and 4-fold higher than its downstream metabolite VAN; in COU-fed cells, 4HB accumulated 3-fold higher than its upstream metabolite 4-hydroxybenzoaldehyde and its downstream metabolite protochatechuate (PCA) was undetectable. When VAN was the carbon source, the PCA level was 25-fold smaller than in *P. putida* fed with 4HB. Based on quantification of intracellular metabolite levels, bottlenecks were identified at four metabolic nodes in the peripheral pathways for different LDA catabolism. The research team aims to overcome these bottlenecks by (1) overexpressing key enzymes involved in the bottlenecks and (2) synthesizing vesicles encapsulating key metabolites and delivering them directly to cells.

Evaluation of the metabolic capabilities of OMVs versus cells can provide insights into the spatial organization of catabolic pathways, providing further insights into potential bottlenecks in the LDA catabolic pathways. To overcome these bottlenecks, genetic tools for the manipulation of OMV biogenesis and enzyme packaging are needed. The current work aims to develop genetic tools in *P. putida* both to induce vesiculation and to target specific enzymes into the OMVs, thus providing additional approaches for engineering LDA bioconversion. To identify genetic targets that influence vesiculation, nine knockout mutants were screened for a hypervesiculation phenotype. Out of these mutants, only two knockouts, both involved in establishing linkages between the outer membrane and peptidoglycan layers, were found to induce biogenesis. Interestingly, high production of OMVs (i.e., 4-fold greater than wildtype) was found to coincide with higher cell membrane permeability and increased cell stress, whereas a moderate increase in OMVs (i.e., 1.5-fold greater than wildtype) did not impact cell performance.

Additionally, a SpyCatcher-SpyTag system was utilized to selectively target specific protein cargo into OMVs, which was demonstrated by an increase in extracellular enzyme activity. These advancements represent strides toward harnessing OMVs as a valuable synthetic biology tool.

Salvachúa, D., et al. "Outer Membrane Vesicles Catabolize Lignin-Derived Aromatic Compounds in *Pseudomonas putida* KT2440," *Proceedings of the National Academy of Sciences* **117**, 9302–10.

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Multichromatic Optogenetic Control of Microbial Coculture Populations for Chemical Production

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Project Goals: The goal of this project is to develop and apply multichromatic optogenetic tools in bacteria and yeast to control coculture populations. Researchers have developed ways to alter microbial growth rates of different strains using blue, dark, red, or near-infrared light by controlling the expression of essential genes or toxin/antitoxin systems in yeast or bacteria, respectively (Wegner et al. 2022; Lalwani et al. 2021**).** Testing different light duty cycles and wavelengths allow for the exploration of optimal microbial population ratios when combined with computational methods for real-time feedback. This work demonstrates the first example of polychromatic control in microbial cocultures to maximize the production of valuable commodity chemicals and biofuels.

Metabolic engineering enables the sustainable production of valuable chemicals, drugs, or biofuels from low-cost renewable substrates by re-wiring microbial metabolism. However, growth defects caused by excessive metabolic burden, suboptimal expression/activity of heterologous enzymes, and endogenous regulatory mechanisms often limit microbial productivities (Wegner et al. 2022). These challenges can be addressed by dividing the labor among different microbes in synthetic microbial communities (consortia or cocultures). Fragmenting biosynthetic pathways among different strains of bacteria or yeast, each producing unique intermediates, significantly reduces the metabolic burden while harnessing special capabilities of different microbial species. This strategy also helps optimize each metabolic module in separate strains, override endogenous regulatory mechanisms, and avoid competing pathways to maximize flux through the biosynthetic pathway of interest. However, stabilizing and controlling the composition of microbial consortia is a formidable challenge (Duncker et al. 2021). While some strains grow quickly, others lag, allowing fast-growing members to take over the culture. Researchers apply optogenetics, where cellular processes are optically controlled using photoswitchable proteins that change shape and function in response to light, to maintain population ratios in cocultures.

Light as gene inducers is nontoxic, tunable, and inexpensive, unlike chemical inducers. However, optogenetic control of microbial populations has only been demonstrated with blue light and only to control the growth rate of one strain in a two-member consortium, in which the optically controlled member grows significantly faster than the uncontrolled (blind) strain. Thus, an optogenetic tool other than blue light is needed for metabolic engineering applications. Researchers established red/near-infrared systems, which enable control of more complex microbial communities, including ones containing members with comparable growth rates. Combining this system with blue lighe circuits provide multichromatic control over bacteria and/or yeast consortia populations (Wegner et al. 2022). In principle, four strains (bacteria/yeast) under different optogenetic circuits (blue, darkness, red, near-infrared) can be combined to afford complex, multichromatic microbial consortia. This allows the engineering of microbial community members to cooperatively produce various commodity chemicals and biofuels, such as isobutanol, possibly maximizing their titers with various light schedules. This work will significantly advance the use of optogenetic control of microbial communities. This is a new paradigm with enormous potential to not only improve the basic understanding of microbial community interactions but also to overcome the obstacles that have stifled the use of synthetic microbial consortia for biotechnological applications. These multichromatic coculture methods are generalizable and can easily be commercialized when significant yield of any important fine chemicals is achieved.

Duncker, K. E., et al. 2021. "Engineered Microbial Consortia: Strategies and Applications," *Microbial Cell Factories* **20**(211).

Lalwani, M. A., et al. 2021. "Optogenetic Control of Microbial Consortia Populations for Chemical Production," *ACS Synthetic Biology* **10**(8), 2015–29.

Wegner, S. A., et al. 2022. "The Bright Frontiers of Microbial Metabolic Optogenetics," *Current Opinion in Chemical Biology* **71**, 102207.

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Response of Soil Microbial Communities to Nitrogen and Phosphorus Input in Sorghum Field

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Project Goals: The interactions between plants and their microbiomes, specifically arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing bacteria (NFB), play a crucial role in supporting host nutrition, immunity, and development. The project aim is to uncover the genetic factors in sorghum that impact the development and effectiveness of microbial communities in different environmental disturbances. Large-scale farming practices commonly depend on water and chemical fertilizers, neglecting the potential advantages of microbiomes in enhancing plant ability to absorb soil water and nutrients and the current implications of irrigation and the utilization of chemical fertilizers on both the economy and the environment. Through a comprehensive analysis, the project's main goal is to identify and characterize sorghum genotypes that can enhance crop productivity and resilience by establishing microbial communities to reduce farmers' reliance on water and chemical fertilizers.

To address the knowledge gap regarding the influence of nutrient availability on microbiomes in multiple compartment niches (rhizosphere, soil, and root), researchers collected samples from an existing genome-wide association study (GWAS) field experiment to examine the differential response of bacteria versus fungi associated with biofuel sorghum genotypes to nitrogen (N) and/or phosphorus (P) inputs. Further, the team will investigate the mechanisms by which sorghum plants maintain a stable presence of AMF and phosphate solubilizing bacteria (PSB) in their root structures, even when the benefits of this symbiotic relationship for nutrient uptake may be compromised. The project will test three hypotheses.

1. The N or P inputs will have distinct impacts on the development of soil microbiome, particularly resulting in a decrease in the abundance of NFB (diazotrophs) and P uptake fungi (AMF, edaphophilic), but the root microbiomes will deviate from these trends. The team postulates that these outcomes may result from biotic filtering of the host, where roots separate the assemblage of microbiome communities associated with sorghum roots from the overall soil community.
2. The co-occurrence networks of AMF and PSB will show higher modularity when P is in limited supply compared to when in luxury supply.
3. The responses to dual N and P input will be less resistant across different compartments, but the strongest resistance will be observed in the root microbiomes. These findings will provide valuable insights for optimizing microbial communities to improve plant health and productivity in both agricultural and ecological contexts.

Assessing the Effect of Nitrogen and Phosphorus Fertilization on Root-Microbial Communities and Yield Response in *Sorghum bicolor*

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Project Goals: This project is designed to identify sorghum genetic factors that drive the formation and function of microbial communities to increase sorghum biomass yield under different environmental conditions. The team will find and characterize sorghum genes and genotypes that can determine optimal crop productivity and durability by creating microbial communities that minimize the need for fertilizer, water, and other inputs.

Plant–microbial interactions play an important role in plant success through processes shaping survival, fitness, and crop yield. The extent to which plants benefit from or are negatively affected by their microbial associations is driven by host and microbial genetics, environmental context, and the interactions among these three contributing factors. As part of a larger research project directed at maximizing the biomass yield of *Sorghum bicolor* through understanding arbuscular mycorrhizal fungal interactions with their host plant, researchers examined *in situ* responses in a diverse sorghum Bioenergy Association Panel to factorially manipulated nitrogen (N) and phosphorus (P) treatments (Brenton et al. 2016). In 2022, 337 sorghum genotypes were grown across 12 blocks (four fertilizer treatments, three replicate blocks) in a previously fallow field in Watkinsville, Ga. Roots were harvested for microbial community analysis after 8 weeks of in-field growth, and aboveground biomass, lodging, and tiller traits were recorded after 5 months.

As expected, strong effects of genotype were found on biomass. N and P treatments, alone, were associated with a minor yet significant reduction in biomass relative to unfertilized controls whereas the combined N and P treatment did not affect yields. Using a subset of three sorghum genotypes with contrasting yield responses across the unfertilized and combined N and P fertilized plots, root-colonizing bacterial and fungal communities were assessed through whole-genome shotgun and targeted amplicon sequencing. As with the aboveground biomass response, there was no observable shift in microbial community composition associated with combined N and P fertilization. The three chosen genotypes also exhibited low intergenotype variability and high intragenotype variability, with individual replicated blocks and therefore field location being the major contributing factor to community composition. Post-harvest N and P soil contents were similar between treatments, though this may be a function of plant nutrient uptake that will be further elucidated by plant nutrient content analysis.

Brenton, Z. W., et al. 2016. "A Genomic Resource for the Development, Improvement, and Exploitation of Sorghum for Bioenergy," *Genetics* **204**(1), 21–33.

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Computer Vision Models Enable Mixed Linear Modeling to Predict Arbuscular Mycorrhizal Fungi Colonization Using Fungal Morphology

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Project Goals: To use systems biology to understand the symbiosis between arbuscular mycorrhizal fungi (AMF) and sorghum.

Computer vision models were used to classify and segment *Sorghum bicolor* root images for AMF structures and to estimate AMF percent colonization as a function of root depth, plant accession, and arbuscule count density in sorghum samples from a recombinant inbred line (RIL) (Govindarajulu et al. 2021) and field samples grown in the greenhouse. A Mask R-CNN model (He et al. 2017) carried out classification and segmentation with high confidence (>0.70 confidence) on AMF structures on both populations. Segmenting the root images enabled calculation of a variety of fungal morphological structural measurements including AMF percent colonization, fungal structure count densities, and average structure size. The fungal morphological measurements of arbuscule structure count density, average arbuscule size, accession as a random effect, and root depth as a fixed effect enabled prediction of AMF percent colonization with a conditional R^2 of at least 0.95 using a mixed linear model (Searle et al. 2009). The intraclass correlation, which measured plant-level variation, was 0.56. Three conclusions were reached: (1) AMF structures were more prevalent in fine sorghum roots in the top 15 cm of soil as a preferred AMF niche; (2) RIL accessions differed in AMF percent colonization; and (3) evidence suggests that plant genotype controls AMF percent colonization through the arbuscule count density in sorghum roots.

Govindarajulu, R., et al. 2021. "Integration of High-density Genetic Mapping with Transcriptome Analysis Uncovers Numerous Agronomic QTL and Reveals Candidate Genes for the Control of Tillering in Sorghum," *G3 Genes/Genomes/Genetics* **11**(2), jkab024. DOI:10.1093/g3journal/jkab024.

He, K., et al. 2017. "Mask R-CNN," *2017 IEEE International Conference on Computer Vision (ICCV), Venice, Italy*, 2980–8. DOI:10.1109/ICCV.2017.322.

Searle, S. R., et al. 2009. *Variance Components*. Eds. John Wiley and Sons. 536 pp.

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Metabolomics Investigates the Impact of Plastic Biodegradation on Mealworm Gut Microbiome

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This study delves into the plastic-degrading prowess of yellow mealworm (*Tenebrio molitor*) gut microbiomes, surpassing known microbial isolates in breaking down plastics like polyethylene and polystyrene without pretreatment. Identified bacterial contributors play a role, but the enhancement

of degradation rates—potentially up to 200% through co-feeding with alternative diets—points to unexplored biodegradation mechanisms. To bridge these gaps, the research team utilized metabolomics and computational analyses to dissect the metabolic pathways involved in plastic degradation. Both gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS) were employed for untargeted metabolomic analysis of mealworm gut samples (e.g., C18 reverse phase LC and Orbitrap MS analysis). Given the complexity of the data, advanced data processing was applied with Compound Discoverer for multivariable statistical analysis. Python was also used for principal component analysis (PCA), complemented by the Kyoto Encyclopedia of Genes and Genomes (KEGG) and MetaboAnalyst for pathway enrichment, providing deep insights into the enzymatic and metabolic underpinnings of this process.

To enhance differentiation between standard (i.e., oat) and plastic diets during sample preparation, microbial samples were separated from large pieces of insect tissue using a 900μm-pore-size filter paper. LC–high resolution MS matching results from samples of three diets (i.e., oat, polystyrene, and polyethylene) reveal 217 matched compounds with 31 compounds achieving match scores exceeding 90. Subsequent metabolite identification highlights that 35% of identified metabolites exhibit significantly smaller normalized peak areas in mealworms consuming polystyrene compared to those on a standard diet ($p<0.05$; fold change >10). Meanwhile, 2% of identified metabolites exhibited significantly larger normalized peak areas. The differential analysis indicates reduced metabolic activity in polystyrene-fed mealworms.

Pathway enrichment analysis, using the KEGG database and MetaboAnalyst 6.0, assesses the impact of metabolites with significant differences. The top-most relevant pathways that may be involved in the mealworm's response to the plastic diet are starch and sucrose metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; arginine biosynthesis; and histidine metabolism. Computational algorithm hierarchical cluster analysis and PCA serve as statistical clustering methods for grouping detected metabolites. While these methods provide statistical insights, the biological implications of the clustering results remain unclear. Currently, the team is working on connecting these clusters with pathway databases and biological network methodologies. This metabolomic research collaborates with Lincoln University, one of the oldest historically black colleges in the United States, and provides modern analytical technology and data science training to students from underprivileged communities.

Future work will focus on three areas: (1) analyzing microbial metabolism changes with different feeding strategies for deeper insights into plastic biodegradation; (2) enhancing data analysis with the reactome pathway database and biological networks; and (3) integrating machine learning with metabolic interaction network analysis to better understand plastic degradation by insect microbial consortia. The research team aims to develop a predictive model for identifying plastic-degrading enzymes in the yellow mealworm gut microbiome using machine learning algorithms and a context-aware enzyme sequence representation. This strategy, inspired by termite gut microbiota research, aims to discover new enzymatic candidates and metabolic pathways crucial for biodegradation, advancing microbial engineering and environmental remediation while illuminating the complex interactions involved in the role of insect microbial consortia in plastic degradation.

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CRISPR Activation of Poplar Target of Rapamycin Genes Improves Nitrogen Use Efficiency and Indicates Possible Functional Divergence

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Project Goals: The goal of this research is to determine the role of signaling mediated by the target of rapamycin complex 1 (TORC1) in nutrient sensing in poplar and elucidate the functional role of genes regulating nutritional responses using CRISPR gene editing, genomics, biochemical, and computational approaches.

Poplar (*Populus* spp.) is an important and sustainable bioenergy and bioproduct plant feedstock, yet scientists' understanding of the pathways and networks governing resource use efficiency is poorly developed. The protein target of rapamycin (TOR) kinase is part of an evolutionally conserved central hub that integrates nutrient, energy, hormone, and biotic and abiotic stress signals by regulating transcription, translation, and metabolism. Compared to other plants species, such as *Arabidopsis* and rice, poplar contains two TOR genes. Using genome editing and computational approaches, this project will elucidate the role of the two genes in nitrogen utilization and determine whether they have diverged in function.

An analysis of a compendium (over 800) of publicly available poplar RNA sequencing datasets indicates that both TOR genes are expressed in a variety of tissues and conditions. TOR expression appears to be coordinately

regulated with the interacting partners LST8 and regulatory-associated protein of TOR (RAPTOR). Studies with each of the two TOR promoter sequences (2.5 kilobyte) fused to a beta-glucuronidase green fluorescent protein reporter gene indicate overlapping patterns of expression for each TOR gene. Using CRISPR activation of each TOR gene individually, or the simultaneous activation of both poplar TOR genes, showed that TOR activation enhanced growth under suboptimal levels of nitrogen fertility. This enhanced growth included significantly greater height, leaf area, stem dry weight, leaf dry weight, and aboveground biomass. Growth analysis suggests that the increase in growth was related to internode initiation/production as opposed to internode elongation. CRISPR TOR activation also revealed phenotypic differences resulting from the activation of the two poplar TOR genes. Interestingly, simultaneous activation of both TOR genes did not result in increased growth or biomass production. The team hypothesizes that differences in the ratio of homo- and heterodimers of TOR may result in different outputs that affects growth, biomass production, and phenotype. This hypothesis is being tested using genome-edited poplars with independent biallelic mutants for each TOR gene; CRISPR-Combo gene edited poplars for the simultaneous production of biallelic knockout mutants of one TOR gene while the respective second TOR gene is activated; and production of expression variants via independent promoter editing of the two poplar TOR genes. Additionally, both constitutive active and dominant negative versions of poplar Rho of plants (ROP) genes corresponding to ROP2 and ROP4 have been generated and transformed into poplar. The effect on nutrient use efficiency and root growth will be presented.

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A Prompt Engineering Approach for Root Confocal Image Segmentation Using the Segment Anything Model

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Project Goals: Establishing a digital anatomical atlas for roots of 11 members of the Brassicaceae family to inform the understanding of gene function and connection between genotype and phenotype. The long-term goal is to develop stress-tolerant oil-seed crops to advance sustainable production of biofuel.

Comparative anatomical studies of diverse plant species are vital for understanding changes in gene functions, such as those involved in solute transport and hormone signaling in plant roots. Through the extraction of quantitative phenotypic data of root cells, researchers can further characterize their response to environmental stimuli, facilitating an in-depth characterization of how genes control root cell development. As the first step for comparative anatomical analysis of root cells, accurate segmentation of individual cells is essential to the analysis of whole root traits. Existing software, such as PlantSeg and MorphographX, utilized neural networks called U-Net for cell wall segmentation. U-Net was a last generation neural network model, which requires training with a large amount of manually labeled confocal images. It is time consuming to retrain the model in order to adapt to new images. Foundational models like the Segment Anything Model (SAM) hold promise across various domains due to its zero-shot learning capability. SAM paired with prompt engineering can reduce the effort and time traditionally consumed in dataset annotation, facilitating a semiautomated training process. In this research, the team evaluated SAM's segmentation capabilities against PlantSeg, a state-of-the-art model for plant cell segmentation. The team found that PlantSeg was able to segment 2,332 plant cells from 20 confocal images of *Arabidopsis* roots. However, 792 such segmentations (34.0% of total segmented cells) were incorrect based on a manual inspection. In contrast, the SAM model without finetuning (Vanilla SAM, or V-SAM) was able to segment 1,052 cells, with only 7.8% incorrectly segmented. Although V-SAM can only find 68.3% of the correct cells found by PlantSeg, this is a surprisingly good performance because V-SAM was never trained on root confocal images. Researchers further finetuned V-SAM with a human prompt of ~1,000 cells by drawing rectangular bounding boxes around cells that were not segmented by V-SAM. Note this is a substantially simpler annotation than the labeling required by U-Net, which requires labeling every pixel of the cell wall from each training image. With the finetuned SAM (f-SAM), researchers were able to segment 2,885 cells correctly from the 20 confocal images, which is 187% of that obtained by PlantSeg. These findings demonstrate the efficiency of SAM in confocal image segmentation, showcasing its adaptability and performance compared to existing tools. By addressing challenges specific to confocal images, this approach offers a robust solution for studying plant structure and dynamics. Overall, this research highlights the potential of foundational models like SAM in specialized domains and underscores the importance of tailored approaches for achieving accurate semantic segmentation in confocal imaging.

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Deciphering Stress-Resilient Growth in Brassicaceae Models: A Comparative Genomics Analysis of Adaptation to Extreme Environments

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Understanding how plants adapt to extreme environments is pivotal for designing novel biofuel crops that avoid competition with conventional food crops and thrive in marginal lands. Researchers' previous studies using extremophyte models in Brassicaceae have suggested that a balance among the regulation of salt and water transport, uninterrupted nutrient acquisition, and the capacity to maintain antioxidant and osmolyte pools plays a critical role in surviving environmental stresses (e.g., high salinity) compared to stress-sensitive sister species. The group expanded its search to include multiple emerging biofuel crops and extremophyte models to test for convergent genomic and transcriptomic features to identify evolutionary strategies preferentially found in plants that are adapted to multiple environmental stresses. Researchers found gene family expansion and positive selection for genes known for their salt responses.

Additionally, the team tested selected candidate genes that were expanded in extremophytes and found evidence for gene subfunctionalization in the extremophyte model, *Schrenkiella parvula*. The results of this research highlight repeated evolutionary innovations in diverse Brassicaceae species that may allow scientists to select genes and pathways that are optimal candidates for improving stress tolerance in diverse biofuel crops better adapted to extreme and changing climates.

Using Biodiversity to Explore the Diversification of Environment-Regulated Growth

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Project Goals:

1. Developing an abscisic acid–responsive element binding factor (ABF) plus gene regulatory network (GRN) to define the diversification of stress-associated gene regulation and train predictive models.
2. Utilizing evolutionary context and species phylogeny to discover novel gene functions.
3. Connecting phenotype to genotype using a modeling framework and advances in the rapid deployment of CRISPR-Cas9-mediated mutagenesis.

Engineering crops for sustainable growth in a rapidly changing environment requires an understanding of how growth-modulating gene regulatory networks are integrated with the environment. By analyzing 10 phylogenetically related species in the Brassicaceae family that occupy diverse ecological niches, researchers have identified species where increasing salinity causes growth reduction or growth promotion. Environment-dependent developmental decisions that are critical for an organism's survival often rely upon the dose-dependent action of signaling molecules such as hormones. Differential growth regulation by one such hormone, abscisic acid (ABA), is the primary mechanism plants use to acclimate to changes in water availability and salinity. The group identified species in which ABA predominantly functions either as a growth repressor or a growth enhancer, suggesting that the differential growth responses observed in response to salinity are partly mediated through this hormone. Comparative anatomical analysis of Brassicaceae species revealed that reduction in meristem cell number contributes to ABA's growth repression while growth promotion involves increases in mature cell length. Further, the team's genetic studies reveal that growth inhibition by ABA is dependent on components of the well-established canonical ABA signaling pathway and is accomplished through the action of ABF transcription factors (TFs). DNA Affinity Purification sequencing (DAP-seq) analysis of the ABF TFs revealed that changes in the ABF-auxin regulatory network can explain differences in the extent of growth repression observed in different species. To further characterize the ABF regulatory network and understand its role in different cellular and environmental contexts, researchers are now exploring the cis-regulatory binding landscape of ABF-interacting transcription factors across 10 species.

Growth promotion by ABA, on the other hand, is regulated by an independent pathway which involves noncanonical ABA receptors, suggesting that the dichotomy in physiological responses to this hormone can be explained by differences at the level of perception as well. The components of the growth-promoting and growth-repressing pathways identified through these comparative growth analyses in evolutionarily related species will help identify hormonal regulators of different growth patterns and provide candidates for tuning growth in agriculturally relevant species.

Funding Statement: This work was funded by the DOE BER program, grants DE-SC0020358 and DE-SC0022985 to J.R.D.

EndoPopulus: Endophyte Inoculation Alters Whole-Plant Physiology and Growth Dynamics of *Populus* Under Nitrogen-Deficient Conditions

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Project Goals: This project aims to understand how microorganisms in the *Populus* microbiome affect host plant health and stress tolerance. The team combines plant physiology experiments under normal, nutrient-limited, and water-limited conditions with field and greenhouse data for crop modeling. The process-based model will guide further examination of microbiological, metabolomic, and transcriptomic data, resulting in a system-level understanding of plant–endophyte interactions from the molecular to the canopy level.

Endophyte inoculation has a potential to improve biofuel crop sustainability by increasing resource use efficiency and stress tolerance, possibly reducing fresh water and fertilizer needs. Previous research demonstrated that Salicaceae endophytes improved water use efficiency in poplar plants under drought conditions (Banan et al. 2024). These endophytes are also known to fix atmospheric nitrogen and alter metabolite biosynthesis, processes that can boost crop productivity. In this study, researchers investigated the morphological changes and resource allocations in poplar trees inoculated with Salicaceae endophytes under nitrogen deficiency at multiple scales ranging from tissue and organ to whole plant. Nisqually-1 *Populus trichocarpa* were mock-inoculated or inoculated with the endophyte consortia as described by Banan et al. (2024). Plants were irrigated daily with either 10 mM (low nitrogen; LN) or 40 mM (high nitrogen; HN) ammonium nitrate (NH_4NO_3) for 150 days. The length of plant shoots and the number of fully expanded leaves were monitored weekly. Upon detecting differences in these data, additional measurements were taken of leaf size and leaf chlorophyll content by a SPAD meter, and their ratios were assessed for entire leaves weekly. Around 60 days after cultivation (DAC), the endophyte-inoculated plants under LN exhibited taller growth and more leaves than uninoculated plants. The inoculated plants maintained higher shoot length up to harvest, but leaf numbers equalized by 150 DAC. Under HN, endophyte inoculation had no effect on shoot length and leaf number. Total leaf area per plant was higher in inoculated plants around 60 DAC, regardless of NH_4NO_3 levels. This difference became larger up to harvest under LN but diminished after 100 DAC under HN. Chlorophyll content followed a similar trend.

Across treatments, the ratio of chlorophyll content to leaf area decreased over time. Under LN, inoculated plants initially had a higher ratio, but declined rapidly after 100 DAC, reaching levels similar to uninoculated plants. Under HN, inoculation did not affect the ratio. Inoculated plants exhibited greater total dry biomass than uninoculated plants, regardless of NH_4NO_3 levels. Under LN, inoculation increased stem dry mass with no effect on root or leaf biomass.

Under HN, stem and leaf biomass trends were similar to LN, but inoculated plants also showed higher root biomass, resulting in a higher root-to-shoot ratio. These results suggest that endophyte inoculation aids plant adaptation to stress, enhancing host productivity and resource use efficiency; larger leaves and increased chlorophyll content suggest this adaptation promotes growth under nitrogen-deficient conditions. These physiological data will be used for process-based modeling to quantify the potential of endophytes to enhance biofuel feedstock production.

Banan, D., et al. 2024. "Endophyte Mediated *Populus trichocarpa* Water Use Efficiency Is Dependent on Time of Day and Plant Water Status," *Phytobiomes Journal.* DOI:10.1094/PBIOMES-11-22-0077-R.

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EndoPopulus: Elucidation of the Roles of Diazotrophic Endophyte Communities in Promoting Productivity and Resilience of *Populus* Through Systems Biology Approaches

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Project Goals: The overall project goal is to move toward an understanding of the holobiont, how plants and the microbial community within them interact in ways that promote the productivity of the whole. Integration of plant physiology data with molecular plant–microbe interactions (multiomics) data from greenhouse and field experiments will allow researchers to develop a systems-level understanding of the genetic and molecular basis for diazotrophic endophytic mutualism in *Populus*. This deeper level of understanding of plant responses will guide construction of microbial communities to optimize the impacts of bioinoculants for environmental sustainability of bioenergy crops.

This project aims to unravel the molecular mechanisms of nitrogen fixation by endophytes of *Populus*. In year 4, researchers published on the intricacies of nitrogenase activity in the aerobic endophyte, *Burkholderia* strain WPB (Sher et al. 2024). Nitrogenase promoter fusions to GFP, as well as NanoSIMS with the $^{15}N_2$-exposed strain, indicated that only a subset of the population actively fixes nitrogen (N). Prominent early signatures of N-fixation activity included polyamines and amino acids, while non-N-fixing cells of the population produced more trehalose and citric acid. Poplar plants within RhizoChips were used to assess nitrogenase gene expression in association with the host plant. WPB nitrogenase activity was particularly strong within nitrosomes of plant root epidermal cells. The wild plant microbiome includes a wide variety of diazotrophic bacterial species, so researchers expanded the research to include *Rahnella aceris* strain WP5; *Azospirillum* sp. 11RA; and *Azorhizobium* strain HT1-9, an endophyte of a Hawaiian lava bed plant. The acetylene reduction assay assessing nitrogenase activity demonstrated that oxygen is required for full activity of 11RA and HT1-9 but that WP5 needed a microaerobic environment. To further elucidate the molecular mechanisms of endophytic nitrogen fixation, researchers have conducted genome-wide transposon-insertion (RB-TnSeq) mutant screening assays to determine genes required for nitrogen fixation at various oxygen levels in WP5 and 11RA. Following up on the initial findings in *Burkholderia* sp. WPB, researchers have constructed additional fluorescently tagged mutants of both WPB and 11RA to further characterize the nitrosome structures. They continued a detailed analysis of the synergy effect discovered in 2020, uncovering requirements for the complex microbial interactions that amplify nitrogenase activity.

Greenhouse- and field-scale experiments were conducted to identify molecular and physiological impacts on plants under nutrient-limited conditions and water-limited conditions. Physiological impacts are presented in an accompanying poster by Sun Woo Chung. Metabolomics analysis revealed an endophyte-mediated metabolic shift in leaf tissues. Specifically, potential roles of specific organic acids, amino acids, and phytohormones and their correlations with physiological parameters were identified. Plant colonization by each endophyte strain will be verified using strain-specific primers in droplet digital PCR. The team constructed nitrogenase mutants to investigate the microbial mechanisms responsible for the plant impacts. In addition to the experiments focused on N-limitation, researchers also initiated more studies on endophyte-mediated phosphate solubilization. Media and protocols are being optimized prior to mechanistic studies.

Sher, A. W., et al. 2024. "Dynamic Nitrogen Fixation in an Aerobic Endophyte of *Populus*," *The ISME Journal*, wrad012. DOI:10.1093/ismejo/wrad012.

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Harnessing Bacterial–Fungal Interactions to Improve Switchgrass Nitrogen Use Efficiency

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Switchgrass (*Panicum virgatum*) is a model perennial crop that can be grown on marginal land to sequester carbon into stable soil organic matter and produce sustainable biofuels. Nitrogen (N) fertilizer maintains switchgrass biomass productivity on marginal lands but excessive fertilization offsets potential carbon sequestration gains by increasing both carbon dioxide and nitrous oxide emissions. To ameliorate

excessive fertilizer, a combination of inoculated and native microbiota can be leveraged to improve switchgrass N management. Free-living diazotrophs are ubiquitous nonsymbiotic bacteria that fix atmospheric N into plant-available ammonium. Free-living diazotrophs are estimated to fix ~47 kg N ha^{-1} in the switchgrass feedstock system, and their activity is stimulated by switchgrass root exudation in the rhizosphere. However, free-living N fixation is difficult to predict because it is controlled by soil edaphic conditions. To limit confounding effects from soil native communities on N fixation, researchers quantified the effects of diazotroph inoculation on switchgrass N using sterile microcosms during a one-week pulse-labeling experiment. Switchgrass seedlings grew in a sterilized, sand-turface (1:1) mixture for 10 weeks under high or low N fertilizer conditions. Subsequently, seedlings were inoculated with either *Azotobacter vinelandii* DJ (0.6 optical density) or sterile, nitrogen-free medium. Directly after diazotroph inoculation, the microcosms were placed into an airtight labeling chamber; the team pulsed 1.0 L of $^{15}N_2$ for seven days before harvesting aboveground biomass to quantify ^{15}N enrichment. There was a significant, interactive effect between N fertilization and diazotroph inoculation. Diazotroph inoculation increased switchgrass total N only under low-N conditions, suggesting that inorganic N significantly impacts N fixation activity ($p < 0.05$). $\delta^{15}N$ enrichment ($\delta^{15}N > 500$ ‰) was only identified in switchgrass inoculated with diazotrophs under low-N conditions ($p < 0.05$).

In addition to free-living diazotrophs, switchgrass associate with arbuscular mycorrhizal fungi (AMF). The AMF symbiont forages for inorganic nutrients beyond the plant's root zone by extending extraradical hyphae deep into the bulk soil. AMF provide up to 55% of inorganic N requirements under low-N conditions. However, plant–AMF mutualism does not occur in a vacuum. Bacteria, including free-living diazotrophs, live along the fungal hyphae. Interactions with N-fixing bacteria could boost plant benefits from AMF symbiosis, yet little work has been done to evaluate synergies among N-fixing bacteria residing on AMF. Building on previous GLBRC work on free-living N fixation in bioenergy crops, the team developed split-pot microcosm systems that enable spatially explicit sampling of AMF hyphal bacterial communities in the greenhouse and the field.

The team identified methodological considerations for characterizing free-living diazotroph establishment and N-fixation activity on AMF. The team will share future research endeavors to quantify nutrient benefits from these interactions via the Bioenergy Cropping System Experiment (BCSE) at Kellogg Biological Station. Ultimately, this research quantifies the contributions of AMF-diazotroph interactions to switchgrass N health, determining if this alternative source of N can ameliorate excessive fertilization on marginal lands.

Integrating Molecular Genetics and Precision Phenotyping to Elucidate the Genetic Basis for Drought Resilience in Sorghum

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Project Goals:

- Overall project objective: To define and functionally characterize genes and pathways related to drought stress tolerance in sorghum and the molecular mechanisms by which these factors drive phenotypic diversity.
- Establish a foundation for deep explorations of gene regulatory networks in sorghum through integrative genomics analyses.
- Enhance understanding of how genotype drives phenotype and environmental adaptation using high-resolution, field-based phenotyping of sorghum mutant collections and a novel diversity panel.
- Map and characterize genes contributing to drought responsive phenotypes in sorghum.
- Experimentally validate predictions of gene function using molecular and genetic assays and targeted gene editing.

Development of the next generation of bioenergy feedstocks will require strategies that utilize resource-limited agricultural lands. This project investigates the innate drought resilience of sorghum (*Sorghum bicolor*), a bioenergy feedstock and cereal crop. Drought is a complex trait and identifying the genes underlying sorghum's innate drought tolerance and how they are regulated in the broader context of the whole plant and its environment requires advanced approaches in genetics, genomics, and phenotyping.

This project leverages a field-based phenotyping infrastructure in Maricopa, Ariz., which provides an exceptional capability for managed stress trials in a hot, arid environment through controlled irrigation. An automated field scanner system collects high-resolution phenotyping data using a variety of sensors throughout the growing season, from seedling establishment to harvest. A chemically mutagenized sorghum population was phenotyped under the field scanner to compare drought-stressed and well-watered plants. Each mutant's genome has been sequenced so sequence variants

can be linked with phenotypes. Being able to assess the genotype-to-phenotype link in response to drought over the life cycle of the plant will facilitate discovery of genes and their functions. A custom diversity panel of sorghum lines was built that maximizes variation in water use efficiencies as well as genetics and geographic origin. Each of these lines have been sequenced through various efforts. This panel was phenotyped under a field scanner and in controlled-environment drought response experiments. Samples were collected for population-level expression analyses. State-of-the-art phenotyping data analytics pipelines have been developed as part of this project and DOE-funded initiatives (see poster by Gonzalez et al.) and are being extended to define stress-related phenotypes at multiple scales. To accelerate mapping of causal loci that underlie mutants of interest, researchers use bulked segregant analysis-seq. So far, researchers have identified candidate genes underlying defects in leaf senescence, shoot and root architecture, and fertility. Regulatory maps generated from diverse sorghum lines in response to stress are being used to nominate gene candidates and place them in the larger context of a drought response network. Finally, an in-house, leaf-based transformation and gene editing pipeline is being used to generate mutant alleles in sorghum for characterizing gene function.

This work will identify control points for enhancing the productivity of bioenergy crops in marginal environments through precision breeding or engineering, thus accelerating the development of improved high-yeilding varieties under limited water resources.

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Analyzing Biotic and Abiotic Stress Responses in Sorghum Using Comprehensive Field Phenomics Data

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https://github.com/phytooracle

https://datacommons.cyverse.org/browse/iplant/home/shared/phytooracle

https://charcoal-dryrot-quantification.streamlit.app/

Project Goals:

- Use the University of Arizona field scanner to gather field phenomics data from EMS-mutagenized sorghum populations and a custom diversity panel, under well-watered and water-limited conditions.
- Develop software and machine learning (ML) models to analyze field phenomics data, quantifying individual plant traits to study plant responses to biotic and abiotic stresses.
- Leverage trait data to investigate genotype–phenotype associations and elucidate gene functions.

Sorghum [*Sorghum bicolor* (L.) Moench], the fifth most cultivated cereal crop, is increasingly grown in the United States due to its adaptability to marginal lands and diverse uses as a food, feed, and biofuel crop (Ndlovu et al. 2022; Yang et al. 2022; Hossain et al. 2022). Expanding sorghum cultivation requires understanding its natural and induced resistance to biotic and abiotic stress. Recent technological advances have resulted in small, low-cost, high-resolution sensors that can rapidly collect phenotypic trait data at regular time intervals in field or greenhouse settings (Li et al. 2020; Sooriyapathirana et al. 2021). Today, high spatial- and temporal-resolution field phenomics data are being collected to extract information on dynamic plant responses to abiotic and biotic stress under real-world field conditions.

The University of Arizona houses the world's largest outdoor plant phenotyping system, the Field Scanner. It uses various sensors to collect plant trait data, including red-green-blue

(RGB), photosystem II chlorophyll fluorescence, thermal imagery, and 3D point clouds. This raw data is processed using PhytoOracle, a collection of scalable, modular pipelines for phenomic data (Gonzalez et al. 2023). The PhytoOracle pipelines facilitate extraction of phenotypic trait data at multiple levels, from whole plants to individual organs. The ML models segment plant point clouds to gather detailed morphological data. This includes traditional shape descriptors like height, volume, and angle. Additionally, topological data analysis (TDA) is used to study subtle shape nuances. Common TDA methods, like persistence diagrams and Euler characteristic curves, capture topological signatures for a more nuanced shape study (Amézquita et al. 2022; Amézquita et al. 2020; Chazal and Michel 2021).

Additionally, ML models are being utilized to identify particular stress factors, including biotic stress. Sorghum, while drought-resistant, is vulnerable to various pathogens, including the destructive soil-borne fungus *Macrophomina phaseolina* (Tassi) Goid. This fungus causes charcoal rot of sorghum (CRS), disrupting the plant's water and nutrient transport, and leading to symptoms often confused with other conditions like drought stress and frost damage. An automated method distinguishing CRS from other stresses could improve selection accuracy, heritability, and genetic gain, ultimately facilitating the development of more resilient crop cultivars. Various ML models trained to identify and quantify CRS in RGB images are available on a web-based application where users can easily analyze their own images: https://charcoal-dryrot-quantification.streamlit.app/.

In studying both abiotic and biotic stress factors, this research seeks to enhance crop productivity by pinpointing variations in stress resilience. Through the application of fine-scale phenotyping, this research contributes to the development of improved, climate-resilient crop varieties.

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Amézquita, E. J., et al. 2022. "Measuring Hidden Phenotype: Quantifying the Shape of Barley Seeds Using the Euler Characteristic Transform," *Silico Plants* **4**, diab033.

Chazal, F., and B. Michel. 2021. "An Introduction to Topological Data Analysis: Fundamental and Practical Aspects for Data Scientists," *Frontiers in Artificial Intelligence* **4**, 667963.

Gonzalez, E. M., et al. 2023. "PhytoOracle: Scalable, Modular Phenomics Data Processing Pipelines," *Frontiers in Plant Science* **14**, 1112973.

Hossain, Md. S., et al. 2022. "Sorghum: A Prospective Crop for Climatic Vulnerability, Food and Nutritional Security," *Journal of Agriculture and Food Research* **8**, 100300.

Li, B., et al. 2020. "Phenomics-Based GWAS Analysis Reveals the Genetic Architecture for Drought Resistance in Cotton," *Plant Biotechnology Journal* **18**, 2533–44.

Ndlovu, E., et al. 2022. "Morpho-Physiological Effects of Moisture, Heat, and Combined Stresses on Sorghum bicolor [Moench (L.)] and its Acclimation Mechanisms," *Plant Stress* **2**, 100018.

Sooriyapathirana, S. D. S. S., et al. 2021. "Photosynthetic Phenomics of Field- and Greenhouse-Grown Amaranths vs. Sensory and Species Delimits," *Plant Phenomics*, 1–13.

Yang, Q., et al. 2022. "Genetic Analysis of Seed Traits in *Sorghum bicolor* that Affect the Human Gut Microbiome," *Nature Communication* **13**, 5641.

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Optimizing Enzymes for Plastic Upcycling Using Machine Learning Design and High-Throughput Experiments

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Project Goals: Researchers aim to create new and optimized polyethylene terephthalate-depolymerizing enzymes (PETases) useful for industrial application.

- Aim 1: Design novel PETases that are significantly different (25 to 65+ mutations) from known PET-depolymerizing enzymes and contain unique properties useful for performant enzymatic PET recycling and upcycling. Introducing many simultaneous mutations, while maintaining function, will enable researchers to more efficiently search for altered properties that depend on primary amino acid sequence.
- Aim 2: Optimize previously described PETases by testing millions of mutagenized variants using directed evolution. Starting with existing functional PETases and exploring small changes in many distinct sequences using a novel ultra-high-throughput functional assay, researchers will optimize enzymes with improved properties by varying experimental conditions.
- Aim 3: Characterize performance metrics of new and optimized PETases in detail including solvent tolerance, stability, catalytic rate, and substrate promiscuity.

Plastics use is ubiquitous in the modern world, and polyethylene terephthalate (PET) is one of the most abundantly produced plastics (and the most highly produced polyester), with ~65 million metric tons manufactured annually. To the consumer, PET is likely most recognizable as the plastic used to make beverage bottles. Like many plastics, traditional mechanical or chemical means of PET deconstruction and upcycling are costly and inefficient. Recently, biological enzymes capable of breaking down PET into its basic building blocks (terephthalic acid and ethylene glycol) have garnered significant attention as an attractive means of dealing with the plastics problem. These enzymes are currently undergoing pilot studies for implementation in enzyme-based recycling. However, there are significant limitations to current enzymes, including the need to perform costly pre-processing of plastics waste before the enzymes are able to work. Further optimization of these enzymes is necessary to make the process profitable and thereby incentivize commercialization of this biology-based green recycling technology.

In this work, researchers apply recent advances in artificial intelligence and machine learning to design new versions of enzymes capable of breaking down PET. From a multiple sequence alignment of natural homologs of PETase, researchers derive models of the protein family. These models are used to design new sequences with a large number of mutations (5 to 20% of positions) that are in parsimony with the homologs yet are distinct in their primary sequence. In addition to this new computational methodology for sequence design, the team also rationally introduced point mutations known to promote PETase enzyme stability or activity. To test design variants, the team developed a high-throughput robotic testing platform capable of enzyme purification and characterization of hundreds of putative PETase enzymes across a range of 16 conditions (e.g., pH, temperature, and substrate).

Using the new experimental platform, researchers have now tested ~800 designed PETases that contain ~10 to 70 amino acid changes relative to their wildtype chassis. The majority of these enzymes have *bona fide* PETase activity, deconstructing PET into terephthalic acid and monomeric mono-2-hydroxyethyl terephthalate. Additional rounds of design on top of these already performant designs tend to further enhance activity. In addition to demonstrating drastically increased activity compared to the wildtype from which they derived, many of the designs also demonstrate higher activity than the top PETases in the literature (e.g., LCC-ICCG). Individual designs tended to be performant in very specific conditions (i.e., amorphous film / 70°C / pH 4.5) rather than showing broad performance across multiple conditions. From this large diverse set of designed PETases, researchers generally have one or more variants that best the top-published PETases in every condition. Ongoing work is aimed at probing the mechanistic consequences of both individual and sets of mutations, furthering a predictive understanding of these performant enzymes. The simultaneous application of combinations of designs in future work will probe for synergy and additivity towards breakdown of diverse PET substrates.

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Systems Framework to Enhance the Potential of *Camelina* as Oilseed Crop

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https://camregbase.org/

Project Goals: This project will: (1) characterize the genetic variation, gene expression and chromatin accessibility across *Camelina* varieties and growth conditions and (2) develop the tools to understand and manipulate *Camelina* gene expression. An important goal is to identify key genes and genomic regions to target inbreeding efforts to enhance productivity while providing the research community with a number of tools to understand and manipulate *Camelina* gene expression. An important project objective is to make available to the community a new set of tools and resources for *Camelina* that have been limited in the past to model plant systems.

The adoption of *Camelina sativa* as an industrial oilseed crop hinges on being able to increase its modest yield. This is in part constrained by limited knowledge of the gene regulatory networks responsible for plant growth and environmental responses, and by a poor understanding of the genetic diversity and gene content across *Camelina* accessions. To address these shortcomings, researchers have started developing a *Camelina* transcription factor (TF) open reading frame (ORF) collection (TFome) that will accelerate the discovery of protein–DNA interactions. For example, *Camelina* TF ORFs were used for carrying out DNA affinity purification with high-throughput sequencing (DAP-seq) towards the identification of candidate fatty acid regulators. Researchers have standardized conditions for embryo and seed Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) to identify accessible chromatin regions and compare them among the three subgenomes that comprise the hexaploid genome of *Camelina sativa*. Researchers are also completing sequencing of a new version of the *Camelina* variety Suneson. Finally, researchers are standardizing conditions for the growth and transformation of the diploid and tetraploid precursors of hexaploid varieties to facilitate crop resynthesis and introduction of novel genetic diversity.

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Surveying, Culturing, and Sequencing Root Microbiomes Associated with Switchgrass, a Native North American Biofuel Feedstock

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Root-associated bacteria play a central role in plant health, affecting ecosystem processes and impacting agricultural sustainability. The group aims to better understand the interactions between root-associated bacterial communities (i.e., microbiota) and switchgrass, a biofuel feedstock C_4 grass native to North America. Researchers have surveyed root microbiota from a population of resequenced, natural switchgrass accessions growing across its native range (Edwards et al. 2023). The team found that switchgrass root microbiomes are dependent upon the field site in which they grow. Within field sites, researchers found that the abundance of many different microbes depends on the genetic background of the host plant. Genome-wide association studies (GWAS) identified several host genetic loci of interest which may impact microbiome assembly.

The above study helped researchers understand how host plants modulate their bacterial communities, yet researchers still know little about the bacterial strains that colonize switchgrass, their genomic information, or the interactions they have with plants and other members of the microbial community. In this study, the team used a high-throughput bacterial isolation approach to culture members of the switchgrass microbiome across genotypes. Researchers are using a full-genome sequencing approach to identify the bacterial isolates in the collection, so far sequencing over 3,500 genomes. Researchers present the phylogenetic diversity of the culture collection and how it relates to field surveying efforts, the catalogue and diversity of the culture collection's biosynthetic gene clusters, and an example of how synthetic communities can be formed and inoculated on plants to evaluate assembly patterns. Researchers envision these bacterial isolates with their corresponding genomes to be an important resource for the switchgrass microbiome community.

Edwards, J., et al. 2023. "Genetic Determinants of Switchgrass Root-Associated Microbiota in Field Sites Spanning Its Natural Range," *Current Biology* **33**(10), 1926–38. DOI:10.1016/j.cub.2023.03.078.

Extracting Switchgrass Features Through Minirhizotron and Hyperspectral Image Processing

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Project Goals: This team's goal is to develop computer vision software pipelines for efficient analysis of minirhizotron and hyperspectral images of switchgrass.

Both minirhizotrons and unmanned aerial vehicles (UAVs) can provide a massive amount of image data on plants like switchgrass (*Panicum virgatum*), a potential source of biofuel. However, manual analysis of these images is time-consuming. Researchers focus on developing computer vision software pipelines to segment the images (i.e., classify pixels based on their respective imagery) and accurately quantify data for these segmentations and raw images.

Minirhizotrons allow researchers to track the growth of the same plant roots over time. For any automated analysis, it

will be necessary to align the images so that calculations from pixel differences are accurate. Researchers use the Binary Robust Invariant Scalable Keypoints (BRISK) algorithm for feature detection (Leutenegger et al. 2011), and then random sample consensus (RANSAC) to calculate a homography between matched points and align two images from different dates. Minirhizotrons provide eight color images of the roots at different depths. Researchers experiment with aligning raw minirhizotron images versus segmented images (where roots have been identified) and aligning each level separately versus all at once (when images have been stitched together). Additionally, the group demonstrates an analysis pipeline for UAV hyperspectral data of individual switchgrass plants. This pipeline produces both individual segmentations of switchgrass plants and extractions of vegetation indices for the respective plants using the hyperspectral data and segmentations. The team first obtains segmentations of individual plants using a combination of Sparsity Promoting Iterated Constrained Endmembers (SPICE) and manually inputting keypoints for each plant (Zare and Gader 2007). Then, the team applies the watershed algorithm to assign boundary labels for each plant from the binary SPICE output. The team's minirhizotron analysis pipeline is able to identify a change in biomass over time, and alignment results are promising. The hyperspectral analysis pipeline calculates all vegetation indices after performing radiance, reflectance, and orthorectification processing and stitching together all data cubes. The group's combined aim with this research is to expedite data collection and analysis for biologists.

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Zare, A., and P. Gader. 2007. "SPICE: A Sparsity Promoting Iterated Constrained Endmember Extraction Algorithm with Applications to Landmine Detection from Hyperspectral Imagery," *Proceedings of SPIE* **6553**. DOI:10.1117/12.722595.

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Using Finlay-Wilkinson Regression to Analyze Genotype–Environment Interaction for Biomass from Switchgrass Field Trials

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Switchgrass is a perennial warm season C_4 grass native to much of North America and a promising biofuel feedstock candidate. It is common in most prairies and exhibits extensive variability and adaptation across its range, especially related to latitude and precipitation gradients. Much of this variability is associated with evolved southern lowland and northern upland ecotypes.

This study utilized switchgrass biomass data from a structured mapping population and a diversity panel collected across 10 common gardens over multiple years. These field trial datasets were analyzed using the Finlay-Wilkinson regression (FW) approach to explore the genetic architecture of general vigor and environmental sensitivity. The FW method involves regressing the performance of each genotype against environmental means in a two-step procedure. The first step computes average plant performance at each site–year combination as a metric of environmental quality. The second step estimates the intercept and slope of each genotype regressed against the ordered environmental mean. The slope of the regression is a measure for adaptability and the intercept is a measure for general performance. The research team obtained an intercept, slope, and posterior standard deviation associated with the two parameters for each genotype of the two populations.

For the genetic mapping population, R/qtl2 software was used for quantitative trait locus (QTL) mapping while taking into consideration relatedness and individual weights. Individual weights in this case were calculated as $n/(SD^2)$, where n is the number of occurrences of that genotype

across all the environments and SD is the posterior standard deviation for that genotype obtained from the FW regression. The team identified 23 QTLs associated with the intercept with the most significant QTL on chromosome 5N at marker position 84.03623 centimorgans (cM), and 11 QTLs associated with slope with the most significant QTL on chromosome 3N at marker position 77.91787 cM. For the Atlantic diversity panel, ASRgwas software was used to fit a linear mixed genome-wide association study model including a single nucleotide polymorphism (SNP)-based kinship matrix to control for population structure. A total of 4,128 SNPs were identified ($p<5e-04$) for the intercept, with most significant SNPs showing signal on chromosomes 1N and 7K. For the slope, 2,725 SNPs were identified with the significant SNP primarily localized on chromosomes 5K and 7K. The extensive field trial dataset and analyses reveal several genomic regions and candidate genes impacting general vigor and environmental sensitivity. These data indicate direct strategies for improving high performing switchgrass cultivars across continental-scale environmental variation.

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Identification of Regulatory Mechanisms Underlying Cell Differentiation in Sorghum Biomass

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Project Goals: Researchers plan to alter the genetic regulation of the cellular developmental programs that generate the vegetative tissues of sorghum, with the aim to increase the proportion of cells that are less recalcitrant to biomass deconstruction.

Plant biomass is comprised of distinct cell types, which largely determine its physical and chemical properties, and hence, its recalcitrance to biomass processing aimed at generating fermentable sugars that microbes can convert to biofuels. The walls of parenchyma cells in the stalks of maize [*Zea mays* (L.)] and sorghum [*Sorghum bicolor* (L.) Moench] can be broken down using milder pretreatment conditions and with lower cellulase loadings than the lignified cells present in the outer rind of the stalk. The different cell types within the sorghum plant develop from undifferentiated meristem cells through variation in the spatiotemporal expression of regulatory genes that control structural genes. The understanding of the role of specific genes and their regulation in this developmental process is incomplete. Uncovering the function of the complete ensemble of genes involved in the differentiation and maturation of the cells that make up sorghum biomass creates the opportunity to manipulate its cellular composition, impacting its physical and chemical properties and, consequently, its value for bioenergy.

This research team is applying single-cell genome and transcriptome analysis of the sorghum shoot apex and stem to identify the function of genes involved in differentiating cells that determine biomass composition. Researchers have identified the internode in developing sorghum stems of 30-day-old plants that displays a developmental gradient, whereby the top of the internode is undifferentiated and the bottom of the internode contains parenchyma, sclerenchyma, and protoxylem cells. Nuclei isolated from dissected internodes have been subjected to single-nucleus RNA sequencing using the 10×Genomics platform. Uniform Manifold Approximation and Projection (UMAP) was used to identify clusters of nuclei with similar expression profiles hypothesized to represent different stages of development of the different cell types. In the next phase, inferred cell lineage trajectories involved in the development of the cellular components of biomass will be explored to discover their regulators. Specific objectives are to (1) define the function of each gene (including specific members within gene families) with respect to the development of the main cell types that determine sorghum biomass and its cell-wall composition; (2) construct cellular lineages that give rise to each cell type that composes biomass (from the shoot apical and vascular cambium meristem cells to cells in the stem), and identify genes and cis-regulatory elements that contribute to the lineage progression; (3) categorize the function of gene and associated cis-regulatory components for their relevance in the control of cellular lineages that lead to each cell type; and (4) validate multiple targets in isolation and in parallel to confirm their role in biomass development and their potential for enhancing biomass yield and its properties.

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Natural Diversity Screening, Assay Development, and Characterization of Nylon-6 Enzymatic Depolymerization

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Project Goals: Motivated by the achievements in biocatalytic polyethylene terephthalate (PET) recycling, there is a growing interest in investigating the enzymatic recycling of various other manmade polymers. Polyamides (nylons) have emerged as a logical focus due to the extensive range of naturally occurring amide-active enzymes. In this pursuit, researchers aimed to assess a selection of biocatalysts for their propensity for nylon-6 hydrolysis. The team assessed 40 potential nylon-deconstructing enzymes (nylonases) for their ability to depolymerize solid nylon-6 films. Initially considering enzymes with various catalytic mechanisms, such as nylon oligomer hydrolases, amidases, serine hydrolases, and proteases, researchers also strategically thermostabilized some of the most promising candidates to enhance nylon-6 hydrolysis at high temperatures. The testing of such a range of enzymes should allow researchers to select the best candidate biocatalysts for further interrogation.

Approach and Activities: A high-throughput liquid chromatography with tandem mass spectrometry (LC-MS/MS) method was devised to analyze the products of nylon-6 hydrolysis reactions, simultaneously identifying and quantifying eight potential polymer deconstruction products from a single sample. The 40 potential nylonases were then assessed in time-course reactions spanning 40 to 70°C using nylon-6 film as the substrate and the described LC-MS/MS–based analytical method to quantify the extent of nylon deconstruction. These activities were coupled with rigorous assessments of the nylon substrate pre- and post-enzymatic deconstruction using a range of materials characterization techniques such as differential scanning calorimetry, thermal gravimetric analysis, gel permeation chromatography, and scanning electron microscopy. The best candidate enzymes were then examined more rigorously by altering reaction pH, substrate loadings, and enzyme loadings.

Results and Lessons Learned: Analysis of the products following enzymatic hydrolysis of solid nylon-6 unveiled notable differences in product selectivity among the studied enzyme types. Despite extensive testing, all the nylonases showed low depolymerization extents, hinting at the rarity of robust nylon deconstruction activity amongst natural enzymes. Within the examined enzyme set, a rationally thermostabilized N-terminal nucleophile (Ntn) hydrolase, NylCK-TS, exhibited the highest activity, and was suitable for depolymerization reactions up to 80°C. However, this enzyme only deconstructed 0.7 wt% of a nylon-6 film with reactions levelling off after 7 days. Inability to restart reactions after adding fresh enzyme led the team to hypothesize a substrate-based limitation in further nylon-6 deconstruction, possibly due to the lack of remaining enzyme-accessible amide bonds. In conclusion, this research expands the knowledge of nylonase activity distribution among diverse enzyme types, highlights the promise of Ntn hydrolases for deeper exploration, and identifies crucial pathways for advancing the enzymatic depolymerization of nylon-6. These pathways include further enzyme engineering, refining product selectivity, and augmenting polymer accessibility.

Discovering Transcriptional Regulators of Photosynthesis in Energy Sorghum to Improve Productivity

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https://photosynthesis.web.illinois.edu

Project Goals: This research aims to identify and investigate the transcription factors involved in the regulation of photosynthesis in energy sorghum. The major goal of this project is to model and validate gene regulatory networks and integrate with physiological data to reveal transcription factors that can alleviate the loss of photosynthetic efficiency in lower canopy leaves. This information will allow ranking of transcription factors by importance and thus, will guide future design strategies for developing energy sorghum cultivars with improved photosynthetic light-use efficiency and overall productivity.

C_4 grasses such as energy sorghum (*Sorghum bicolor*) have great potential for both carbon sequestration and as feedstocks for biofuels and building materials. However, in contrast to what is typically observed for other plants, sorghum belongs to a clade of C_4 species that has undergone a maladaptive loss of photosynthetic efficiency in self-shaded leaves within the canopy. Current models predict that this

loss results in a 15 to 20% reduction in potential productivity (Pignon et al. 2017). Specifically, most plants have evolved to dynamically tune their photosynthetic machinery by shifting the stoichiometry of proteins involved in the light reactions of photosynthesis to maintain a high maximum absolute quantum efficiency of carbon dioxide assimilation ($\Phi CO_{2,max}$) in the shade. Work has shown that the lower self-shaded leaves from C_4 bioenergy crops (bioenergy sorghum, *Miscanthus*, and maize) do not retain a high $\Phi CO_{2,max}$ compared to their upper sun-exposed leaves; this change is due to the light environment rather than leaf age (Collison et al. 2020; Pignon et al. 2017). Variation in the severity of this $\Phi CO_{2,max}$ loss between sorghum cultivars suggests that this maladaptive trait may be the result of difference in the expression of one or more genes (Jaikumar et al. 2021). This is supported by recent greenhouse experiments where researchers showed that the phenotype is reversible by moving plants from shaded to light environments. The team hypothesizes that genes influencing $\Phi CO_{2,max}$ will have expression patterns that correspond to measurable changes in photosynthetic traits and that researchers will be able to identify these genes by comparing changes in expression in response to the light environment across energy sorghum cultivars and canopy positions.

Therefore, the team will have collected gene expression and physiological data on photosynthetic traits such as $\Phi CO_{2,max}$ across light conditions from different sorghum cultivars in the field and greenhouse. Since transcription factors (TFs) are key regulators of gene expression in response to environmental stimuli, such as changes in light intensity and quality, the project expects that TF expression is important to the maladaptive loss of photosynthetic efficiency. To identify key TFs, researchers are building gene regulatory networks that integrate gene expression and photosynthetic trait data. The project will improve the accuracy of these networks by including TF gene targets identified using a new *in planta* method. Identifying the cause of photosynthetic inefficiency in shaded energy sorghum canopies and engineering solutions to restore the 15 to 20% loss in productivity and enhance yield will improve the overall potential of this bioenergy crop to meet growing needs for energy security.

Collison, R. F., et al. 2020. "Light, Not Age, Underlies the Maladaptation of Maize and Miscanthus Photosynthesis to Self-Shading," *Frontiers in Plant Science* **11**, 783. DOI:10.3389/fpls.2020.00783.

Jaikumar, N. S., et al. 2021. "Can Improved Canopy Light Transmission Ameliorate Loss of Photosynthetic Efficiency in the Shade? An Investigation of Natural Variation in Sorghum bicolor," *Journal of Experimental Botany* **72**(13), 4965–80. DOI:10.1093/jxb/erab176.

Pignon, C. P., et al. 2017. "Loss of Photosynthetic Efficiency in the Shade. An Achilles Heel for the Dense Modern Stands of Our Most Productive C4 Crops?" *Journal of Experimental Botany* **68**(2), 335–45. DOI:10.1093/jxb/erw456.

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Deciphering Genetic and Physiological Mechanisms of Nitrogen Use Efficiency in Camelina

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Project Goals: Camelina (*Camelina sativa*) is a Brassica oilseed crop that has great potential to become a sustainable source of bioenergy in the United States. However, its low nitrogen use efficiency (NUE) and seed and oil yield compared to other major oilseed crops hinder its potential. The goal of this project is to decipher the genetic and physiological mechanisms that determine NUE and oilseed yield during the most critical processes of the camelina life cycle: (1) how camelina, in partnership with soil microbes, maximizes its ability to absorb and assimilate nitrogen (N) into vegetative biomass and (2) upon the transition to reproductive growth, how N is efficiently remobilized from senescing tissues (leaves and silicles) into sinks (seeds) to optimize yield potential by increasing seed size and enhancing oil synthesis.

Enhancing oilseed yield with minimum N fertilizer input is a major pathway towards sustainable production of camelina oils. Researchers aim to obtain a systems-level understanding of genetic and physiological mechanisms that may be used to increase NUE and improve agronomic and seed traits in camelina.

First, field experiments were conducted to evaluate the genetic diversity of camelina performance and camelina responses to low and high N levels, and to determine the heritability of agronomic traits contributing to NUE. Evaluation of 20 selected varieties indicated significant genotypic variation in plant height, biomass, seed yield, and oil characteristics in both years of 2022 and 2023. While most varieties consistently demonstrated higher yields and oil content

under high N conditions, their responses to N fertilization differed, and several varieties showed low responses to N fertilization. Moreover, the consistently high heritability of traits and N uptake indicate that genetic variation plays a predominant role in shaping these characteristics over environmental factors. The field studies therefore demonstrated a wide variation of key agronomic traits in camelina, but also varied NUE. Camelina lines with different responses to N input were selected for further studies to decipher the genetic and physiological mechanisms of NUE.

Second, genomics approaches were used to isolate genes that contribute to oil yield and NUE in camelina. A chromosome-level genome was assembled, based on which single nucleotide polymorphism and insertion/deletion markers were developed from resequenced genomes of the diversity panel consisting of 212 accessions of *C. sativa* (Li et al. 2021). Agronomic traits including biomass, seed weight, seed oil content, and fatty acid composition were measured by growing in fields at two levels of N fertilization. Genome wide association studies identified quantitative trait loci (QTLs) for the above traits in both N treatments. Focusing on seed oil content, researchers found three QTL regions that explained 12 to 18% of the phenotype variance under low N conditions. To identify candidate genes within the QTLs, researchers analyzed transcriptomes of developing seeds in two camelina lines that differ significantly in their oil contents. A sucrose transporter was chosen as a candidate gene for further functional studies.

Third, N remobilization experiments were conducted to identify physiological mechanisms and genes controlling NUE in camelina. N use of one variety (Suneson) was analyzed by growing plants hydroponically under either high N (6.5 mM) or low N (0.65 mM). N was either removed from the nutrient solution at anthesis or continued until plant maturity (four treatments). N removal led to efficient remobilization from aboveground tissues to seeds, with N concentrations decreasing from ~4% to ~0.5% of tissue dry mass. N remobilization also occurred under high N–continued, but >2% remained at maturity, leading to substantial loss with plant residue. Seed N was at least 3% for all treatments, indicating that this concentration is necessary for viable seeds. In all treatments except high N–continued, the largest fraction of plant N is in seeds at maturity, demonstrating efficient N remobilization. Transcriptomics will be used to identify genes controlling N remobilization efficiency under the four different treatments.

Li, H., et al. 2021. "Genetic Dissection of Natural Variation in Oilseed Traits of Camelina by Whole-Genome Resequencing and QTL Mapping," *Plant Genome* **14**, e20110.

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The Root Microbiome of Camelina: From Structure to Function

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Camelina (*Camelina sativa* L.) is an oilseed crop being developed as a biofuel crop for dryland agriculture. This team is investigating the role of the root microbiome in plant health, especially in relation to nutrient uptake, disease, and drought. Researchers sampled soil from 33 locations in four precipitation/cropping zones in Eastern Washington and grew cultivar Suneson in each soil in the greenhouse. Amplicon sequencing was used to describe the bulk soil, rhizosphere, and endosphere bacterial and fungal communities. Plant compartment, cropping system zone, and location had significant effects on microbial composition. The group identified the core rhizosphere communities of bacteria (several Actinobacteria including *Aeromicrobium* and *Marmoricola*, as well as the genera *Rhizobium, Clostridium*, and *Sphingomonas*) and fungi (*Pseudogymnoascus*, *Fusarium,* and *Mortierella*). Researchers are currently sequencing metagenomes from rhizosphere communities of 10 camelina lines grown under high and low nitrogen (N) conditions to identify how soil N shifts the communities on the root.

More than 400 camelina-associated bacterial isolates were screened for growth promotion on camelina under normal and low N conditions. Eleven bacterial isolates were shown to promote elongation of primary roots under low N levels. Two bacterial strains *Paraburkholderia tropica* (isolates FMD144 and FMD568) showed consistent root growth promotion. One bacterial strain, *Pseudomonas mediterranea* isolate FMD348, inhibited the growth of camelina under all N levels. Co-inoculation of camelina with FMD348 and FMD144 revealed that strain 348 dominated the interaction and caused root inhibition, suppressing the growth-promoting effect of strain FMD144. Interestingly, in a more complex bacteria–bacteria interaction in which FMD348 was co-inoculated with a bacterial community of either 21 isolates or the 11 beneficial isolates, root growth promotion was observed and growth inhibition by FMD348 was

suppressed. The bacterial effect on camelina growth can be positively or negatively influenced by other bacteria in the community.

A diverse set of 33 bacteria selected from a larger collection of over 3,000 camelina-associated isolates were profiled by exometabolomics to determine what these bacteria consume and produce. They were cultured on Northen Lab Defined Medium, a diverse, defined media with over 60 compounds including sugars, organic acids, amino acids, and diverse nitrogenous cofactors and vitamins. Spent media was collected in late exponential phase and analyzed using liquid chromatography–tandem mass spectrometry. Almost every compound in the media was significantly reduced by at least one isolate; however, growth rates and consumption profiles varied greatly across the collection. Production of potential secondary metabolites produced by the cultures also varied greatly across the collection. This data is being analyzed to understand how these microbes are recruited by camelina and interact with one another and will aid in the targeted isolation of future isolates.

Metabolic Modeling and Genetic Engineering of Enhanced Anaerobic Microbial Ethylene Synthesis

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Project Goals: To develop robust and optimized anaerobic ethylene pathways in photosynthetic and lignocellulosic bacteria for high-yield conversion of renewable carbon dioxide (CO^2) and lignocellulose into bioethylene. This will be accomplished by:

1. Bioinformatically mining and experimentally screening methylthioalkane reductase homologs, S-adenosyl-L-methionine hydrolase homologs, and alcohol dehydrogenase homologs from cultivated and uncultivated organisms to identify functional enzymes that enhance ethylene yields.
2. Constructing and employing predictive systems-level models of ethylene production. This project will use a physics-based *Rhodospirillum rubrum* model to predict enzymes that participate in competing or supporting pathways and are thus targets for selection studies to increase ethylene yields.
3. Metabolically engineering bacteria for enhanced, sustained ethylene production from CO_2 and lignocellulose. The project will assemble the best-performing genes under control of optimized active transcription elements on a modular DNA fragment in a combinatorial manner with guidance from predictive models (see goal 2).

Previously, the research team detailed a pathway in the phototrophic bacterium *R. rubrum* that produces ethylene in the absence of oxygen from methionine and ATP (North et al. 2020). Traditional ethylene production involves energy-intensive cracking of petroleum fossil fuels to meet the 300 million metric ton annual demand. Thus, a sustainable microbial platform for the renewable production of ethylene is urgently needed. The goal of this project is to optimize this anaerobic ethylene production pathway.

Enzyme Screens: Methylthio-alkane reductase genes and other nitrogenase-like genes identified from microbial genomes were synthesized and assembled by the DOE Joint Genome Institute DNA synthesis program such that each set contained a NifB, NifH, NifD, and NifK homolog. Sequences were screened for activity in an *R. rubrum* deletion strain devoid of native methylthio-alkane reductase (North et al. 2020). Sequences from the methylthio-alkane reductase phylogenetic clade were active for reduction of volatile organic sulfur compounds of the form R-S-CH_3 if *R. rubrum* could couple electron transfer from cell redox carriers to the NifH homolog. Strikingly, proteins encoded by nitrogenase-like sequences from other phylogenetic clades of unknown function showed no methylthio-alkane reductase activity and showed no activity toward other methylated compounds like methylated amines, sulfates, or phosphates. Thus, the functions of many nitrogenase-like clades remain unknown.

Physics-Based Modeling: During photoheterotrophic growth on organic substrates, purple non-sulfur bacteria like *R. rubrum* acquire electrons by multiple means and store them as reduced electron carriers. The ratio of oxidized to reduced electron carriers [e.g., ratio of $NAD(P)^+$:NAD(P)H)] is difficult to predict but essential for thermokinetic modeling and predicting the targeted metabolic engineering needed to increase ethylene synthesis. Using physics-based models that capture mass-action kinetics consistent with the thermodynamics of reactions and pathways, a range of redox conditions for photoheterotrophic growth was evaluated (King et al. 2023; Cannon et al. 2024). Modeling results and experimental measurements of macromolecule levels (DNA, RNA, proteins, and fatty acids; North et al. 2020) indicate that cellular redox poise results in large-scale changes in biosynthetic pathway activity. The model, which agrees with experimental measurements of macromolecule ratios of cells growing on different carbon substrates, indicates that the dynamics of nucleotide versus lipid and protein production is likely a significant mechanism balancing cellular oxidation and reduction.

Metabolic Engineering: In *R. rubrum,* ethylene production involves 12 genes and nine primary reactions, plus pathways supporting ATP, acetyl-CoA, and NAD(P)H synthesis. Previously, the research team identified high-performing S-adenosyl-L-methionine (SAM) hydrolases, isomerases, aldolases, and methylthio-alkane reductases that individually increase ethylene production. Combining these elements on a mobile plasmid under modest to high levels of constitutive expression results in a 30,000-fold increase in ethylene yields from 0.01 to 300 µmol/g dry cell weight. When antibiotics are included to retain plasmid selection, yields further increase to 1,000 µmol/g dry cell weight. Additional genetic tools are needed to integrate multiple pathway elements onto the chromosome, as four to five pathway elements on a plasmid represents the largest plasmid size *R. rubrum* can stably incorporate.

Cannon, W.R., et. al. 2024. "Redox Poise during Rhodospirillum rubrum Phototrophic Growth Drives Large-scale Changes in Macromolecular Synthesis Pathways," arXiv:2401.04862 [q-bio.MN]. DOI:10.48550/arXiv.2401.04862.

King, E., et al. 2023. "An Approach to Learn Regulation to Maximize Growth and Entropy Production Rates in Metabolism," *Frontiers in Systems Biology* **3**, 981866. DOI:10.3389/fsysb.2023.981866.

North, J. A., et al. 2020. "A Nitrogenase-like Enzyme System Catalyzes Methionine, Ethylene, and Methane Biogenesis," *Science* **369**(6507), 1094–8. DOI:10.1126/science.abb6310.

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Cell-Free Systems Biology: Characterizing Pyruvate Metabolism of *Clostridium thermocellum* with a Three-Enzyme Cascade Reaction

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Project Goals: The overall goal of the project is to develop tools to improve systems-level understanding of metabolism in nonmodel organisms, such as *Clostridium thermocellum*, and use that understanding to increase product titer.

Genetic approaches have been traditionally used to understand microbial metabolism, but this process can be slow in nonmodel organisms with limited genetic tools. An alternative approach is to study metabolism directly in the cell lysate. This avoids the need for genetic tools, and is routinely used to study individual enzymatic reactions, but is not generally used to study systems-level properties of metabolism. Here, the researchers demonstrate a new approach they call "cell-free systems biology" where they use well-characterized enzymes and multi-enzyme cascades to serve as sources or sinks of intermediate metabolites. This allows isolation of metabolic subnetworks and the study of their systems-level properties. To demonstrate this, the research team worked with a three-enzyme cascade reaction that converts pyruvate to 2,3-butanediol. Although it has been previously used in cell-free systems, its pH-dependence was not well characterized, limiting its utility as a sink for pyruvate. The research team showed that improved proton accounting allowed better prediction of pH changes, and that active pH control allowed 2,3-butanediol titers of up to 1.1 M (189 g/L) from acetoin and 1.6 M (144 g/L) from pyruvate. The improved proton accounting provided a crucial insight: preventing the escape of carbon dioxide from the system largely eliminated the need for active pH control, dramatically simplifying experimental setup. Researchers then used this cascade reaction to understand limits to product formation in *C. thermocellum*, an organism with potential applications for cellulosic biofuel production. This team showed that the fate of pyruvate is largely controlled by electron availability and that reactions upstream of pyruvate limit overall product formation.

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Population Genomic Differentiation of the Ectomycorrhizal Fungus *Suillus pungens* Along a Climate Gradient

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Project Goals: This project examines genomic and functional variation among ectomycorrhizal fungi along a natural climate gradient and consequences for host adaptation and ecosystem function.

Dispersal limitations and geographic barriers can influence microbial population structure and gene flow at the landscape level, resulting in divergent genotypes and regional endemism. However, the drivers and genetic basis of population differentiation across large spatial scales, particularly among mycorrhizal fungi, remains poorly understood. This

study investigates the population structure of *Suillus pungens*, an ectomycorrhizal fungus endemic to the California coast and a host-specialist to *Pinus muricata* and *Pinus radiata.* The research team performed whole-genome sequencing on 70 individuals collected across a latitudinal and 4-fold precipitation gradient. The team used a combination of paired-end sequencing on the Illumina NextSeq 500 System (with a 2 x 150 base pair read length) and MiSeq (2 x 250 base pair read length) and produced a total average read depth of 6. Using an annotated reference genome, gene variant bioinformatic approaches (GATK) were employed, resulting in identification of 541,091 single-nucleotide polymorphisms (SNPs) across these 70 individuals. Significant population genetic structure was found among northern and southern populations as well as highly differentiated host-associated genotypes. Of these SNPs, a strong functional signature of adaptation was identified, with southern populations enriched in genes involved in cell signaling and membrane fluidity, a potential adaptation to drought stress. These results provide some of the first genomic evidence for local adaptation within ectomycorrhizal species and show that barriers to gene flow can develop over relatively small spatial scales. Future work will explore how this degree of local adaptation by ectomycorrhizal fungi contributes to host stress tolerance or affects ecosystem function.

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Enhanced Resistance Pines for Improved Renewable Biofuel and Chemical Production

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Project Goals: The team's goal is to genetically increase constitutive terpene defenses of loblolly and slash pine to enhance protection against pests and pathogens and simultaneously expand terpene supplies for renewable biofuels and chemicals.

The constitutive and inducible oleoresin defense network in loblolly (*Pinus taeda*) and slash (*Pinus elliottii* var. *elliottii*) pines provides physical and chemical resistance to insects and pathogens, and the chemical composition of oleoresin can be used as a renewable source of biofuels harvested directly from live tree stems. Increasing pine terpenes is well aligned with the needs of the developing bioeconomy. The southeastern United States currently hosts the world's largest biomass supply chain, annually delivering 17% of global wood products, and has the potential to expand the U.S. pine chemicals industry by increasing biofuels from pine terpenes, which is currently limited by relatively low average wood terpene content. The team's focus is to increase constitutive terpene production to enhance loblolly and slash pine resistance to pests and pathogens and to simultaneously increase biofuel feedstocks in these commercial pine species.

Pine terpenes evolved as a primary chemical and physical defense system and are a main component of a durable, quantitative defense mechanism against pests and pathogens. Previous research demonstrated that terpene defense traits are under genetic control and behave as quantitative traits. They used genetic engineering to validate 12 genes that can significantly increase wood terpene content. In Objective 1, this project is integrating existing and new genome-wide association study (GWAS) genetic results with RNA expression, quantitative trait loci (QTL) mapping, and allele frequency information in known high-oleoresin flow selections and researchers' breeding populations to discover and validate loblolly and slash pine alleles and genes that are important for resistance.

GWAS analyses of constitutive oleoresin flow, wood diterpenoid content, and resin canal number with ~83,000 biallelic single-nucleotide polymorphisms (SNPs) were completed for the project's Comparing Clonal Lines ON Experimental Sites (CCLONES) population. Constitutive and inducible oleoresin flow along with mono- and diterpene content were completed and resin canal number is in progress for the team's Allele Discovery of Economic Pine Traits 2 (ADEPT2) population. In the ADEPT2 population, researchers simultaneously measured constitutive and induced oleoresin flow after treating clones with methyljasmonate (MeJA). While the goal is to increase constitutive terpene defenses, the group used MeJA to induce defense responses to identify the genes and genetic architecture of resinosis. In the ADEPT2 population, researchers found the clonal repeatability of constitutive oleoresin flow and inducible oleoresin flow to be 0.31, suggesting these traits are under moderate genetic control. In the ADEPT2 population researchers observed a strong genetic correlation (0.82) between induced and constitutive oleoresin flow, suggesting the genetic architecture of these traits is strongly shared. Researchers conducted association analyses with constitutive and inducible oleoresin flow, wood monoterpene content and composition, and diterpenoid content. The content was obtained in the ADEPT2 population using linear mixed models and multilocus linear mixed models in ASRgwas and Genome Association and Prediction Integrated Tool packages using two sets of SNP markers totaling

~2.28 million biallelic SNPs. After controlling for multiple testing, researchers identified 146 significant SNPs ($p<0.05$) for 10 oleoresin traits, including constitutive oleoresin flow and monoterpene composition and content. Two of the significant SNPs for wood limonene content are in an α-pinene synthase gene. To validate significant SNPs, the team quantified oleoresin flow in a pseudo-backcross population between one F1 slash x loblolly hybrid genotype backcrossed to slash and loblolly genotypes. The team is now completing genotyping of 982 individuals with the Pita50k chip for future QTL mapping.

To identify genes regulating resin duct differentiation and function, researchers induced new axial resin canal formation in the cambial meristem by applying MeJA, a known inducer of traumatic resin canal formation in the Pinaceae family. Researchers conducted a time-course experiment by creating 78 RNA sequencing (RNAseq) libraries on vascular cambial-zone tissue collected on days 0, 1 to 14, 17, and 21 after MeJA treatment. Pooled libraries were sequenced to a 30x read depth with the NovaSeq Illumina next-generation sequencing platform. The reads were mapped to an improved *de novo* loblolly pine transcriptome that includes 64,671 genes that researchers constructed with existing expressed sequence tags contigs, Pacific Biosciences reads, and predicted transcripts from loblolly pine reference genome v2.01. DESeq2 analysis identified significantly 1,890 up and 4,634 down differentially expressed genes across the time course compared with wildtype controls. With these 6,524 differentially expressed genes, the team created a Predictive Expression Network (PEN) using iterative Random Forest Leave One Out Prediction to illustrate higher order interactions between genes and to determine the gene-to-gene relationships that are the most highly predictive of each other. To identify and prioritize genes across the PEN that are involved in axial resin canal formation, researchers applied random walk with restart (RWR) algorithms based on a set of literature-curated seed genes that included known orthologous regulators of xylem formation and development, which are suppressed while resin canal formation is increased. The RWR approaches allowed the team to identify mechanistically associated genes that did not appear in GWAS due to a lack of statistical power or genetic variation but are still important components of resinosis. This identified 119 transcripts in the top 200, based on lines of evidence. Researchers are continuing to annotate the network to identify genes whose expression supports involvement in resin canal formation and terpene synthesis.

In Objective 2, the group is using information from Objective 1 to accelerate breeding for increased resistance in loblolly and slash pines through marker assisted introgression, and will develop and test genomic selection models to accelerate breeding of resistant slash pine.

Funding Statement: This research was supported by the DOE Office of Science, BER program–U.S. Department of Agriculture Biomass Feedstocks DE-SC0 019099.

Improving Candidate Gene Discovery by Combining Multiple Genetic Mapping Datasets

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Project Goals:

1. Exploit domain knowledge on phosphorus action in roots to identify strong predictors for phosphorus data and employ an XGBoost model to predict phosphorus levels for ~2,000 georeferenced sorghum landraces distributed in Africa.
2. Perform an environmental genome-wide association study (GWAS) in those landraces that have already been genotyped using predicted phosphorus data as phenotypes for the GWAS analysis.
3. Characterize the genetic architecture of lipid content during the early stages of sorghum development using the Sorghum Association Panel (SAP). Researchers will perform a GWAS on lipid content under low temperature and low phosphorus.
4. Develop algorithms that incorporate all the different types of information the group collects (i.e., metabolite levels, GWAS candidate genes, and selection signals) to improve researchers' ability to detect signals of small effects and increase their confidence in the selection of candidate genes. The algorithms and pipelines developed here will be made available to the community as R packages.

Phosphorus (P) is one of the three primary nutrients in commercial fertilizers, essential for plant growth and development. Excessive use of P-rich fertilizers in agriculture leads to leaching and runoff to water bodies, harming aquatic life. The limited global reserves of rock P and water pollution make it necessary to find a sustainable solution that ensures the proper utilization of P, minimizing leaching and runoff. Landrace varieties adapted in soils with varying levels of P availability likely possess unique genetic mechanisms to cope with P scarcity.

An environmental GWAS using these genotypes with georeferenced accessions presents a potential for identifying candidate genes. Employing GWAS, the group seeks to

identify genes and pathways associated with P in plants that will help researchers overcome these obstacles. Central to GWAS success is accurate phenotype measurement. To this end, researchers have developed an XGBoost model predicting P availability in the soil using domain-based knowledge, surpassing current models in prediction accuracy and capability to discern lower-end values. Utilizing high-dimensional genetic datasets of georeferenced *Sorghum bicolor* in Africa, the team will conduct an environmental GWAS using the team's new P availability data. The team will use a linear regression–based p-value combination method (MAGMA) to aggregate multiple small effects on a gene-based level. The group's previous study has identified lipid variations, specifically phosphatidylcholine, in maize adapted to low P conditions in the Mexican highlands.

Additionally, researchers have obtained lipid profiles for 400 SAP accessions grown in normal and low-P conditions. Analyzing the corresponding lipid dynamics will play a significant role in understanding P utilization. Subsequently, a GWAS will be conducted focusing on these identified candidate lipids. By employing the Cauchy Combination test to combine the findings from both the P GWAS and lipid GWAS, this team aims to reevaluate and redefine the order of gene importance. This integrative analysis will facilitate the identification of candidates that are linked to both lipids and P efficiency.

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Physical and Molecular Responses of Pennycress (*Thlaspi arvense L.*) to Waterlogging

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https://www.pennycressresilience.org/

Project Goals: This project employs evolutionary and computational genomic approaches to identify key genetic variants that have enabled *Thlaspi arvense L.* (Field Pennycress; pennycress) to locally adapt and colonize all temperate regions of the world. This, combined with knowledge of metabolic and cellular networks derived from first principles, guides precise laboratory efforts to create and select high-resilience lines, both from arrays of random mutagenesis and by employing cutting-edge CRISPR genome editing techniques. This project will deliver speed-breeding methods and high-resilience mutants inspired by natural adaptations and newly formulated biological principles into a wide range of commercial pennycress varieties to precisely adapt them to desired local environments.

Pennycress is a winter annual with extreme cold hardiness and seed oil properties desirable for sustainable aviation fuel production. Integration of pennycress as an off-season biofuel cash crop into Midwest corn and soybean rotations could lead to the production of 1 billion liters of seed oil annually, therefore boosting farmer revenue and offsetting carbon emissions. Pennycress fields are vulnerable to heavy spring precipitation events, which can lead to waterlogged soils where the root system is submerged under water. However, it is unknown if growth, development, or yield of pennycress is affected by waterlogging at the reproductive developmental stage, which occurs during April. This work aimed to characterize the morphological and transcriptomic responses of pennycress under 1 week of waterlogging during the reproductive stage. This was done with two core research pennycress lines: MN106 and SP32-10. One week of waterlogging at the reproductive stage under controlled conditions significantly impacted SP32-10 traits at harvest, including a reduction in shoot and root dry weight, total seed count, and total seed weight, whereas MN106 yield traits were not significantly affected. Therefore, natural phenotypic variation in waterlogging responses existed between these two pennycress accessions, so they were further investigated to determine the transcriptomic responses contributing to waterlogging tolerance. Twice as many genes were differentially expressed between waterlogged and control roots in MN106 (3,424 genes) compared to SP32-10 (1,767 genes) after 1 week of waterlogging at the reproductive stage. Functional enrichment analysis of upregulated, differentially expressed genes in both lines revealed gene ontology terms associated with hypoxia and decreased oxygen, including genes involved in alcoholic fermentation and glycolysis. Compared to SP32-10, MN106 waterlogged roots exhibited stronger upregulation of genes involved in hypoxia and glycolysis, as well as strong downregulation of cell wall biogenesis genes. This indicates a better ability of MN106 to respond to the severe energy crisis invoked by waterlogging, possibly by strongly activating anaerobic responses and limiting growth to conserve energy. Lastly, to functionally test the roles of *HRE2* and *SUS1* genes in waterlogging tolerance, which were highly expressed in waterlogged roots, ethyl methanesulfonate mutagenesis lines were waterlogged at the reproductive stage for 1 week. These two mutant lines were highly sensitive to waterlogging and had significantly reduced seed weight compared to controls, supporting the involvement of these genes in the response and adaptation to low-oxygen stress in pennycress. This research has identified phenotypic and transcriptomic variation in waterlogging responses in pennycress, leading to the identification of candidate genes and pathways involved in waterlogging

tolerance. This work provides a foundation for understanding root waterlogging responses in pennycress, which will be useful for breeding climate-resilient pennycress varieties to improve yield following flood events and to grow pennycress on marginal lands.

Combs-Giroir, R., and A. R. Gschwend. 2024. "Physical and Molecular Responses to Flooding in Brassicaceae," *Environmental and Experimental Botany* **216**, 105664. DOI:10.1016/j.envexpbot.2024.105664.

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Reduced Environmental Plasticity in Pennycress Improves Responses to Competition and Climate Change

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https://www.pennycressresilience.org/

Project Goals: The Integrated Pennycress Resilience Project (IPReP) employs evolutionary and computational genomic approaches to identify key genetic variants that have enabled *Thlaspi arvense L.* (Field Pennycress; pennycress) to locally adapt to and colonize all temperate regions of the world. This, combined with knowledge of metabolic and cellular networks derived from first principles, guides precise laboratory efforts to create and select high-resilience lines, both from arrays of random mutagenesis and by employing cutting-edge CRISPR genome editing techniques. This project will deliver speed-breeding methods and high-resilience mutants inspired by natural adaptations and newly formulated biological principles into a wide range of commercial pennycress varieties to precisely adapt them to desired local environments.

Pennycress, an emergent winter annual bioenergy oilseed cover crop, is under development to be grown in the Midwest during typical fallow periods. Pennycress varieties can yield over 1,680 kg/hectare (1,500 lb/acre) of seeds, producing 600 liters/hectare (65 gallons/acre) of oil annually without competing with food crops. However, crucial work remains to domesticate and optimize pennycress for incorporation into present cropping systems and its resilience to climate change. For example, interseeding into standing fields in late fall leads to shade-induced responses in pennycress. Similarly, higher temperatures during fall planting cause seedlings to elongate, resulting in poor stand establishment. Researchers have established that pennycress is shade- and heat-intolerant and elongates in response to these stresses. Excessive elongation creates a cyclical dilemma in which the elongated plants increasingly shade their neighbors, further inducing retaliatory elongation responses to outgrow neighboring plants. These adaptive morphogenic changes are undesirable in cropping systems, as elongated plants establish poorly, are more prone to lodging, and reduce yields. Researchers are using the knowledgebase from *Arabidopsis thaliana* to manipulate genes in the phytochrome signaling pathway to improve resilience to shade present during interseeding and increasing winter and spring temperatures. Evaluation of CRISPR and ethyl methanesulfonate alleles of phytochrome-interacting factor 7 (*pif7*) show that *pif7* mutants have reduced organ elongation and retain a compact rosette when exposed to shade, elevated temperature, and combined stresses while maintaining yield and desirable phenotypes such as earlier flowering in stress conditions. By lowering elongation responses to shade and elevated temperature, researchers aim to increase pennycress ground cover when grown at high densities, reduce shade-induced responses when interseeded into standing crops, and reduce elongation in response to higher temperatures. In addition to light responses, researchers are addressing the freezing tolerance of pennycress by examining the role of fatty acid modifications on winter survival and in response to chilling and freezing stress. Furthermore, researchers have used CRISPR and gene editing to target *CAMTA* and *CBF* family genes and RNAseq, metabolomics, and fatty acid analysis to examine global changes to low temperatures. Future work will determine if these changes to shade and temperature responses improve the performance, productivity, and resilience of pennycress in the field.

Funding Statement: This research is supported by the DOE Office of Science, BER program, GSP grant number DE-SC0021286.

Functional Analysis of Genes Encoding Ubiquitin Proteasome System Components Affecting Poplar Wood Traits

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Project Goals: Wood vessel trait candidate genes coding for E3 ligase proteins will be functionally characterized through the creation of CRISPR-Cas9 mutants, TurboID proximity labeling, and drought and abscisic acid (ABA) treatments to study gene expression, protein ubiquitination, degradation, and abundance in poplar wood–forming tissues.

Angiosperm wood contains highly lignified tube-like cells called vessel elements, which provide a pathway for the upward movement of water under tension. The dimensions and distribution of vessels in wood (i.e., wood anatomy) affect water transport and growth rates, as well as susceptibility to hydraulic failure during drought. Despite their crucial role in determining the hydraulic physiology of trees, the genetic regulation of vessel element anatomical traits is poorly understood. In a dosage-dependent genome-wide screen, researchers detected a significant correlation between height-adjusted mean vessel diameter and frequency. A subsequent gene coexpression network analysis on poplar wood–forming tissues found that height-corrected vessel frequency was significantly correlated to genes that code for E3 ubiquitin ligase, key components of the ubiquitin proteasome system (UPS). The team selected vessel trait–related E3 ligase candidates for further functional characterization of ubiquitin-proteasome regulation in poplar wood forming tissue. To achieve this goal, CRISPR-Cas9 mutants targeting poplar E3 ligase candidate genes have been generated to assess alterations in wood phenotype, gene expression, protein abundance, and ubiquitinomes. Additionally, TurboID transgenic lines are being generated to elucidate protein interacting partners for the candidate proteins through proximity labeling. In anticipation of poplar E3 ligase datasets, researchers examined the interactome of similar components in *Arabidopsis* transgenic lines subjected to drought and ABA treatments, revealing substantial treatment-induced alterations in the UPS compared to controls. These approaches aim to enhance understanding of the role of the ubiquitin-proteasome system in wood formation, vessel trait variation, and tree responses to environmental stressors.

Plastic Degradation and Upcycling by the Gut Microbiome of Yellow Mealworms

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Project Goals: This project discovers and reconstructs the plastic degradation pathways distributed across the gut microbiome of yellow mealworms (larvae of *Tenebrio molitor*) to develop enhanced capabilities for biologically based polymer recycling.

Globally, more than 25 million tons of low-density polyethylene (LDPE) are produced annually, which form significant polluting waste streams at end of life due to a lack of robust infrastructure for mechanical or chemical recycling. To address this need, researchers pursue biological strategies for LDPE deconstruction. Researchers focus on the microbiomes of yellow mealworms or the larvae of *T. molitor* as they degrade plastics more rapidly than microbial isolates and do not require clean plastics or pretreatment like mechanical and chemical recycling. While bacterial community members have been identified, the specific pathways responsible for biodegradation remain to be elucidated.

Early project progress confirmed that mealworm diet strongly impacted plastics consumption rates and enriched plastics-modifying microbes. More recently, researchers validated that gut extracts containing this rich microbial community degrade the molecular weight of LDPE films by more than 3 orders of magnitude within a week, confirming the importance of the gut microbiome in plastics degradation. Researchers are currently characterizing these communities via integrated omics analyses at all levels.

To understand the mechanisms by which these microbes degrade LDPE, researchers have isolated a collection of mealworm gut microbes, which were screened for growth on LDPE as a primary carbon source. Faster-growing isolates are strongly correlated with increased oxidation of LDPE films via carbonyl insertion, consistent with the hypothesis of promiscuous alkane metabolism for plastics degradation. These isolates were also statistically enriched in peroxidases, oxidases, and other proposed LDPE-degrading enzymes (PE-ases). Heterologous expression and *in vitro* testing of candidate PE-ases from these isolates on LDPE films yielded a positive hit that introduces aldehyde groups in LDPE films. *In situ* inhibition of this enzyme class in the mealworm

gut is sufficient to abolish PE deconstruction, suggesting a role in activating plastics degradation. *In vitro* testing, phylogenetic analysis, and simulated protein folding studies of other enzymes that belong to this functional class revealed a subclade of active PE-ases that structurally diverges from non-PE-degrading enzymes in the same class.

In parallel, researchers also develop photoreactive chemical probes that resemble PE oligomers and have affinity for plastics-degrading enzymes for more high-throughput discovery. PE probes were validated by evaluating the proteins they bind in commensal isolates from the yellow mealworm. The team is beginning to identify secreted and intracellular proteins that researchers anticipate enable efficient plastics degradation. The studies also demonstrate the potential for live-cell probe labeling to enable cell sorting and isolation, which researchers are now pursuing to create functionally enriched plastics-degrading consortia.

This work has validated the ability of *T. molitor* gut extracts to deconstruct PE and developed strong evidence for a critical microbial enzyme that activates PE substrates for deconstruction. Researchers have also developed significant omics resources, microbial isolates, and consortia that are beginning to reveal novel strategies and protein structural motifs for enhanced PE degradation. This project generates comprehensive systems-level insight into plastics degradation pathways and aims to develop design rules for synthetic consortia and enzymes enriched in plastics degradation activity.

Funding Statement: This material is based upon work supported by the DOE Office of Science, BER program, GSP, under award number DE-SC0022018. A portion of this research will be performed under the Facilities Integrating Collaborations for User Science (FICUS) initiative and use resources at the DOE Joint Genome Institute and the Environmental Molecular Sciences Laboratory, which are DOE Office of Science User Facilities. Both facilities are sponsored by the BER program and operated under contracts DE-AC02-05CH11231 (JGI) and DE-AC05-76RL01830 (EMSL).

Improving Bioprocess Robustness by Cellular Noise Engineering

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Project Goals: The overall goal of this project is to enhance the robustness of biofuel-producing microbes in adverse and fluctuating environments, such as media containing toxic hydrolysates or elevated temperatures, by introducing cellular noise in gene expression. The project's approach involves the identification of factors in the transcription process that increase cellular noise and the deployment of such factors to generate cells exhibiting increased cellular noise. The project uses modeling and single-cell analysis workflows to engineer *Yarrowia lipolytica* variants that can tolerate, grow, and efficiently synthesize biofuel precursors under steady-state, albeit dynamically stressful, conditions. Overall, the team anticipates that strains with optimal levels of cellular noise will also exhibit robustness that maintains production under time-varying stresses.

Robustness represents a system-level trait that allows cell populations to maintain function under adverse and fluctuating environments. When observed at the cellular or subcellular level, an isogenic cell population exhibits increased cell-to-cell variability, or noise, even under steady-state conditions. In this context, isogenic cells can undergo division of labor, with some expressing the pathways that enable them to continue functioning in a new environment. This concept guides the project in developing workflows for introducing and manipulating cellular noise to enhance cellular tolerance to environmental stressors. Focus has been placed on the construction of *Y. lipolytica* strains with the double phenotype of tolerance and high lipid productivity. In its first steps on cellular noise engineering, the project refined gene-editing toolboxes that can deterministically vary the level of cellular noise in protein expression levels. Accordingly, the team introduced two to eight tandem upstream activating sequences to the p*TEF* promoter. The synthetic hybrid promoters were placed upstream of a green fluorescent protein, fused into plasmids, and stably integrated into the genome of *Y. lipolytica*. Each transformant was screened separately by flow cytometry to categorize them into expression and noise levels. The impact of activators or repressors on the promoters was likewise investigated. As a next step, researchers introduced key genes that play a significant role in viability at varying inhibitor levels. To this end, the team applied rational design to develop a cellulosic oil *Y. lipolytica* strain that is tolerant to the primary lignocellulosic inhibitor furfural. To enable tolerance to furfural, researchers constructed *Y. lipolytica* overexpressing an endogenous aldehyde dehydrogenase that converts furfural to the less toxic furoic acid. The project finally evaluated front-runner *Y. lipolytica* strains under both stressful and non-stressful conditions to quantify the effects of noise and expression levels on furfural tolerance. The team has also identified the mechanisms and related gene targets that could enable *Y. lipolytica* to withstand elevated temperatures, which form the next cellular-noise engineering goal in this project.

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Transcriptional Profiling of Winter-Regulated Genes in *Populus trichocarpa*

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Project Goals: This project's goals include (1) identification of genes that are specifically expressed during winter dormancy, (2) identifying their transcriptional regulators using expression quantitative trait loci (eQTL) mapping, (3) transgenic validation of candidate genes, and (4) incorporation of workflows into the DOE Systems Biology Knowledgebase (KBase) platform.

Xylem and bark samples were collected from 800 natural accessions of mature black cottonwood (*Populus trichocarpa*) planted in a common garden in Clatskanie, Oreg. To investigate the molecular mechanisms involved in cell survival during dormancy, samples were collected at two time points: December 2022 when trees were dormant and July 2023 during the growing season.

Initially, a small set of core samples was collected from approximately 5-year-old field-grown poplar trees established at the University of Tennessee experimental field site in Alcoa, Tenn. These samples were then used to refine the sampling procedure to ensure that (1) the most RNA-rich tissues are collected (bark, cambium, and most recent xylem) to capture transcriptomic responses and (2) both the sampling and subsequent sample processing steps are scalable and suited to study a large population under field conditions.

After this initial optimization, 818 genotypes of the population of natural variants of *P. trichocarpa* were sampled, generating over 2,400 samples across dormancy and growing seasons. To optimize the transcriptomic analysis, a subset of 40 genotypes was selected based on contrasted growth (highest or lowest trunk diameter) and wood properties (highest or lowest wood density). For these genotypes, samples collected in winter were ground and RNA was isolated and sequenced (RNA-seq).

Based on transcriptome sequencing results, workflows were tested, optimized, and established to perform comprehensive analyses to meet project goals. Future efforts include completion of RNA-seq and differential gene expression analyses, eQTL mapping, and incorporation of workflows into the KBase platform.

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Engineering Bacterial Microcompartments in *Clostridium autoethanogenum* to Overcome Bottlenecks in Sustainable Production of Synthetic Rubber

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https://dtelab.northwestern.edu/research/#nanobioreactors

Project Goals: To investigate bacterial microcompartments in *Clostridium autoethanogenum* and engineer them to compartmentalize synthetic metabolic pathways.

One promising route to sustainable bioproduction of fuels and chemicals is the engineering of organisms, such as acetogens, to efficiently convert abundant and low-cost gases containing carbon monoxide or carbon dioxide and hydrogen to desirable, value-added products at high efficiency and low cost. This approach not only provides an avenue for repurposing greenhouse gases (GHG) but also minimizes the use of harsh chemicals and hazardous byproducts common in petroleum-based processes. However, many biochemicals are not yet produced biologically due to roadblocks in the cellular biosynthesis process. These roadblocks can include intermediate toxicity, redox imbalances, and loss of product to off-pathway reactions. In nature, these issues are often alleviated using spatial organization strategies, such as sequestration in organelles. In bacteria, such organization often occurs in protein-based organelles known as bacterial microcompartments (MCPs).

The team will investigate the native regulation, assembly, and function of MCPs in the industrially relevant non-model host *C. autoethanogenum*. In its genome, two unique gene clusters have been identified as putative operons encoding sets of proteins required for MCP formation. These putative operons express a variety of possible MCP shell proteins and encapsulation peptides that target enzymes into the MCP. Team members tested potential inducers of these operons and found that some of these small molecules were consumed by *C. autoethanogenum*; RNA sequencing data

showed that these same small molecules transcriptionally activate the MCP operons. MCP formation in these conditions was corroborated by electron microscopy of *C. autoethanogenum*, which shows distinctive polyhedral shapes within the cells, indicative of MCP formation. The group also used cell-free protein synthesis to produce putative MCP shell proteins from *C. autoethanogenum* and observed self-assembly of large structures, visible under light microscopy.

Beyond understanding the native function of these putative MCP operons, one engineering goal is to sequester key biosynthesis enzymes from two distinct metabolic pathways into MCPs to make compounds involved in rubber production. Specifically, the group aims to showcase the power of enzyme encapsulation in an MCP for reducing toxicity and product losses to side reactions for these pathways. Towards enabling heterologous enzyme encapsulation in these new MCP systems, 16 *C. autoethanogenum* reporter strains were generated with different putative encapsulation peptides fused to superfolder green fluorescent protein (sfGFP). Fluorescence microscopy shows that 11 of these 16 sfGFP-encapsulation peptide fusions exhibit punctate fluorescence upon MCP induction, indicating successful encapsulation of the fluorescent reporter within MCPs. These results demonstrate the potential for encapsulating biosynthesis enzymes and enable the cost-efficient production of chemicals that are currently derived from petroleum.

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Structural Characterization of GT47 Glycosyltransferases in Duckweed to Facilitate Predictive Biology

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Project Goals: The long-term objective is to develop optimized computational and experimental design schema to study plant processes at the systems level to enable precise and reliable prediction of plant gene function. Studies of substrate specificity across the glycosyltransferase 47 (GT47) family will be evaluated through modeling-based predictions and cryoelectron microscopy (cryo-EM) to determine the molecular mechanisms that underlie duckweed cell wall synthesis.

Complex carbohydrates are essential molecules of life responsible for energy supply and diverse cellular functions in all species. GTs facilitate the creation of glycosidic bonds, which are essential for synthesizing intricate carbohydrates that are the building blocks of the carbon stored in plant biomass. One of the team's main goals is to use high-throughput methods to determine sugar nucleotide donor and acceptor substrate specificities for genes encoding GTs to functionally assign them into glycopolymer-specific pathways. The aim is to harness these pathways to reengineer duckweed cell walls for optimized biofuel and feedstock production. These data are being used in a combinatorial approach involving machine learning models to understand and predict substrate specificity: AlphaFold to predict structures of both monomeric and oligomeric GT47 carbohydrate-active enzyme family proteins (Zhang et al. 2023) and cryo-EM to validate the predictions experimentally. The Facilities Integrating Collaborations for User Science (FICUS) program through the Environmental Molecular Sciences Laboratory (EMSL) and DOE Joint Genome Institute (JGI) will help researchers broaden the enzyme library construction to identify GT47 complexes through a combination of plant engineering, mass spectrometry, solid state nuclear magnetic resonance spectroscopy, and structural biology to analyze the protein interaction networks of all GT family members and the integral architecture of the cell wall structure of duckweed. Ultimately, the data generated from this proposal will be used to inform functional studies in a species-agnostic manner to create designer-specified cell wall structures for bioproduction.

Zhang, L., et al. 2023. "Glycosyltransferase Family 47 (GT47) Proteins in Plants and Animals," *Essays in Biochemistry* **67**(3), 639–52. DOI:10.1042/EBC20220152.

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Functional Characterization of GT47 Glycosyltransferases in Duckweed to Facilitate Predictive Biology

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Project Goals: The long-term objective is to develop optimized experimental design schema for utilizing computational prediction and high-throughput functional validation to study plant processes at the systems level and efficiently translate knowledge gained to link genome sequence with gene function.

Duckweeds are fast-growing, aquatic energy crops that produce large amounts of biomass enriched in complex, non-cellulosic carbohydrates that are highly amenable to conversion into fuels and bioproducts. Enzymes called glycosyltransferases (GTs) participate in the biosynthesis of these carbohydrates that enable storage of carbon and energy as glycopolymers. To date, precise functional predictions are extremely difficult or completely unreliable for GTs, as many families are polyspecific. The team is performing family-wide characterization of GT47 enzymes in duckweeds as a model for high-throughput (HTP) functional studies of enzymes involved in carbohydrate metabolism. This family is highly expanded in energy crops and members display diverse substrate specificities, ultimately contributing to the synthesis of almost every class of polysaccharide within biomass (Zhang et al. 2023). Gene functional validation efforts are being performed using a multidisciplinary approach involving enzyme and substrate library construction, HTP biochemical assays, and computational biology. The combined data are being used to populate a machine-learning framework to better enable prediction of plant GT function. The team will also present preliminary work on how regression models, trained on GT sequences, can be used to predict donor specificity, highlighting the need for robust curated data for interpretive machine-learning frameworks. Functional validation achieved through this research project will be used to assign plant gene function and study processes at the systems level to efficiently link genome sequence with function in a feedstock-agnostic manner.

Zhang, L., et al. 2023. "Glycosyltransferase Family 47 (GT47) Proteins in Plants and Animals," *Essays in Biochemistry* **67**(3), 639–52. DOI:10.1042/EBC20220152.

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Phenotypic and Molecular Characterization of Nitrogen-Responsive Genes in Sorghum

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Project Goals: This project will phenotypically and molecularly characterize the 33 existing CRISPR-Cas9-edited nitrogen (N)-responsive genes. Subsequently, the research team will conduct gene editing for the three glutamate-like receptor (GLR) genes in a cluster and GLR-related genes in the N network and generate a population-scale RNA-seq dataset to cross-validate edited genes and identify new gene candidates for further characterization.

The inefficient use of inorganic N fertilizer in crop production increases ecological burdens including biodiversity loss, N leaching into groundwater, and greenhouse gas emissions (e.g., nitrous oxide) which contribute to global warming. Moreover, inorganic N fertilizer stands out as one of the most expensive and energy-intensive agricultural inputs, particularly for sorghum cultivation.

Enhancing sorghum's nitrogen use efficiency (NUE) will not only boost its profitability as an energy crop but also alleviate the environmental burdens associated with its cultivation. To understand the biological basis of this essential macronutrient and ultimately enhance NUE, extensive research has been conducted to reveal processes for N assimilation, transport, and reallocation. Studies in model plant species have identified specialized nitrate transporters, enabling N mobilization processes to be well characterized.

However, N sensing, signaling, and downstream regulatory pathways in crop species remain largely unclear.

Previously, this research team has generated resources and accumulated extensive experiences in N-related research on sorghum. The team conducted transcriptomic analysis, using data collected from sorghum genotype Tx430 grown in different N levels, and edited 33 N-responsive genes using CRISPR-Cas9. The current project has developed a high-throughput phenotyping pipeline to extract N-related phenotypes from the state-of-the-art LemnaTec Greenhouse.

During the summer of 2023, the team collected a variety of manually measured and imagery-based data on the Sorghum Association Panel (SAP; n=330) under both high-N and low-N field conditions. Data included traits related to root morphology and root-associated microbial characteristics. Additionally, the team conducted RNA-seq on three-week-old seedlings from SAP grown in high-N and low-N greenhouse conditions. Currently, the research team is developing statistical models and conducting empirical analyses to integrate multiomics data and provide valuable biological insights. Notably, population genetics and comparative genomics analyses have suggested that a cluster of glutamate-like receptor genes may function as cellular N sensors, activating Ca^{2+}-dependent N signaling—an essential step in the N pathway.

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Systems Biology to Enable Modular Metabolic Engineering of Fatty Acid Production in Cyanobacteria

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Project Goals: The overall objective of this project is to use systems biology to identify metabolic control points and bottlenecks that regulate flux to free fatty acids (FFAs) in cyanobacteria. The central hypothesis is that cyanobacterial lipid metabolism can be modularized into pathways upstream and downstream of the nodal metabolite acetyl-CoA, which can be separately studied and optimized to enhance overall FFA production. The research team plans to test the central hypothesis and accomplish the overall objective of this project by pursuing the following specific aims:

1. Identify upstream metabolic control points regulating acetyl-CoA precursor availability. The working hypothesis is that engineering glycolytic pathways in *Synechococcus* sp. strain PCC 7002 will reveal rate-controlling steps that can be manipulated to maximize acetyl-CoA availability.
2. Assess flux bottlenecks in the downstream fatty acid biosynthesis pathway. The working hypothesis is that multiomics analyses of thioesterase-expressing strains will elucidate regulatory nodes that control FFA production and overall lipid metabolism in strain PCC 7002.

Cyanobacteria, such as the halotolerant strain PCC 7002, are attractive hosts for FFA biofuel precursor production because they produce renewable chemicals directly from photosynthesis, grow in nutrient-poor environments, and readily incorporate genetic modifications. Despite the advantages of cyanobacterial FFA synthesis, production rates are currently too low to support its adoption as a source of renewable fuel.

The overall objective of this project is to enhance cyanobacterial FFA production by identifying and eliminating metabolic bottlenecks upstream and downstream of the FFA building block acetyl-CoA. Upstream of acetyl-CoA, the approach uses a lactate-producing pyruvate sink in an engineered PCC 7002 strain as a model to study the metabolic changes associated with increased flux toward the acetyl-CoA precursor, pyruvate. Applying ^{13}C metabolic flux analysis has revealed that enhanced pyruvate flux in the L-lactate-producing strain is associated with increased flux through the malic enzyme shunt but no change in flux through pyruvate kinase. These data suggest that pyruvate kinase may constitute a metabolic bottleneck limiting overall pyruvate-generating flux.

Downstream of acetyl-CoA, work has centered around the ketosynthase FabH, a putative bottleneck in cyanobacterial FFA biosynthesis previously identified *in vitro*. Heterologous ketosynthase expression was found to enhance cyanobacterial FFA production, but this effect heavily depends upon the level and timing of ketosynthase induction.

This project leverages a suite of systems biology approaches and novel analytical methodologies (e.g., Fast-Pass DESI-MSI for high-throughput screening and AEXpurif for acyl carrier protein analysis) to identify and investigate distinctive FFA production phenotypes. Ultimately, this work will enable integrated strategies that simultaneously address upstream and downstream metabolic bottlenecks to enhance FFA production in cyanobacteria.

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Integrated Experimental Approaches to Understand Bioenergy Crop Productivity Through Rhizosphere Processes

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Project Goals: This project couples novel laboratory and field studies to develop the first predictive model of grass microbiomes based on new mechanistic insights into dynamic plant-microbe interactions in the grasses *Sorghum bicolor* and *Brachypodium distachyon* which improve plant nitrogen (N)-use efficiency (NUE). The results will be used to predict plant mutants and microbial amendments that improve low-input biomass production for laboratory and field studies validation. To achieve this goal, researchers will determine the mechanistic basis of dynamic exudate exchange in the grass rhizosphere with a specific focus on the identification of plant transporters and proteins that regulate root exudate composition. Researchers will also focus on how specific exudates select for beneficial microbes that increase plant biomass and NUE. The team will further develop a predictive plant–microbe model for advancing sustainable bioenergy crops and will predictively shift plant–microbe interactions to enhance plant biomass production and N acquisition from varied N forms.

Microbial amendments are a powerful approach for promoting plant N acquisition, uptake, and cycling using less inputs. Yet, the performance of microbial amendments is highly variable due to the dynamic and complex nature of soil abiotic and biotic interactions. Understanding the factors driving rhizosphere assembly and dynamics, especially when combined with plants with tailored exudates, has the potential to greatly improve the reliable performance of beneficial microbial amendments at lower N levels. The team assessed the potential of a grass rhizosphere synthetic microbiome in promoting *B. distachyon* growth in soils under replete and limited N levels and observed enhanced ability to extract N from soil organic N pools when subjected to limited N conditions. For a more detailed analysis of N cycling in the rhizosphere, including the potential role of root exudates in mediating beneficial microbial interactions, the team grew *B. distachyon* hydroponically in novel fabricated ecosystem devices (EcoFAB 2.0) under three inorganic N forms (nitrate, ammonium, or ammonium nitrate), followed by N starvation. EcoFAB 2.0 achieved low intratreatment data variability and reproducible plant phenotypes. Analyses of exudates with liquid chromatography with tandem mass spectrometry revealed that the three inorganic N forms caused differential exudation, generalized by an increase in amino acids, peptides, and alkaloids. Comparatively, N-deficiency decreased N-containing compounds but increased carbon-rich shikimate phenylpropanoids. Subsequent bioassays with two shikimate phenylpropanoids (shikimic and p-coumaric acids) revealed their distinct capacity to regulate bacterial and plant growth. Given the importance of root exudates in structuring rhizosphere communities, researchers are also investigating transport mechanisms for root exudation, particularly N, by using *B. distachyon* plant mutants. Hydroponic growth of these mutants with knockout N transporters resulted in significant phenotypic and exometabolic changes. Concurrently, researchers are analyzing the microbiome communities of these mutants in calcined clay treated with a field-soil extract to explore the role of root exudation in plant–microbe interactions. Sequencing of rhizosphere and root microbiomes has shown significant changes in bacterial species, indicating that membrane transport engineering can alter plant-root exudates and microbiome composition. Together, these findings advance understanding of the mechanisms that drive plant–microbe interactions to inform the development of more robust microbial amendments for sustainable bioenergy.

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Science Focus Area
Solvent Disruption of Biomass and Biomembranes

Amphiphilic Cosolvents Disrupt the Lateral Structure of Model Biomembranes and Reveal an Unrecognized Mode of Cell Stress

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https://sfa-biofuels.ornl.gov/

Project Goals: This Science Focus Area is developing fundamental knowledge about the ways that solvents change the structures of plant cell walls and microbial membranes. The team's overarching hypothesis is that the partitioning or binding of solvent molecules from the bulk phase to biomass or biomembranes will predict maximal or minimal disruption. Disruption of biological structures comprised of amphiphilic molecules and polymers (e.g., membranes and biomass) is a key step in biomass pretreatments and engineering the ultimate microbial limits in tolerating specific solvents. Researchers integrate the power of world-class neutron scattering capabilities and leadership-class supercomputing facilities available at Oak Ridge National Laboratory (ORNL). These capabilities are complemented by expertise in biodeuteration and biomembranes at ORNL, plant cell wall chemistry at the University of Tennessee, and neutron scattering and membrane biophysics at the University of Cincinnati.

Fuels and value-added chemicals derived from sustainable lignocellulosics are an important part of realizing the future circular bioeconomy. This requires efficient production of biointermediates, including ethanol or n-butanol, which have chaotropic effects on biomolecules leading to their toxicity to fermentative microbes. While these toxic effects can impact many macromolecules, the cellular membrane is especially vulnerable to disruption due to the partitioning of amphiphilic cosolvents into the lipid bilayer. This leads to well-understood impacts on the transverse membrane structure at high cosolvent titers—membrane thinning, destabilization, loss of membrane potential, and eventually, cell death. The effects on lateral biomembrane structure have been less well studied, however. Lateral membrane organization can be understood in analogy to an in-plane phase separation of high and low melting point lipid species, as well as sterols (or their microbial analogs). This results in local membrane regions with different membrane compositions and physical properties. In the biological context, these structures are known as functional membrane microdomains or lipid rafts. Rafts play an important role in many cellular processes due to their role as platforms to sort, colocalize, and assemble membrane proteins.

Researchers propose the hypothesis that amphiphilic cosolvents, such as ethanol and n-butanol, alter or disrupt functional membrane microdomains, leading to an unrecognized mode of cosolvent toxicity and cellular stress at cosolvent concentrations far lower than those which induce membrane disruptions. To support the investigations, researchers developed novel neutron scattering and molecular dynamics simulation strategies to experimentally observe nanoscale changes in membrane structure in living cells.

Researchers demonstrated the direct disruption of a model lipid raft due to the presence of the amphiphilic cosolvent ethanol and elucidated the physical mechanism for the lipid domain disruption (Tan et al. 2023). Subsequently, n-butanol was observed to be more potent in disrupting membrane organization. Researchers have measured an unequal partitioning of n-butanol between the coexisting phases, leading to an increased mismatch in the hydrophobic thickness of these phases, confirmed by neutron-scattering observations. This leads to an increase in domain line tension, which drives minimization of the domain interface to area ratio. These observations are complemented with large-scale simulations replicating the lipid domain, with molecular details about n-butanol partitioning and induced changes to the lipid domain interface.

Researchers also performed small-angle neutron scattering measurements and extensive all-atom simulations on the partitioning of amphiphilic cosolvents (e.g., ethanol, tetrahydrofuran, and n-butanol) in living cell membranes and cell membrane extracts. The team showed direct measurements of cell membrane thinning in living *Bacillus subtilis* in the presence of these cosolvents using neutron scattering, estimated the true partition coefficient of these cosolvents, and interrogated the role of fatty acid supplementation in

* Presenting Author

modulating the cellular response to these solvents. This work represents the first systematic approach of the nanoscale membrane phenotype in fermentative microorganisms. Further validation of this hypothesis will provide a more holistic understanding of solvent–membrane interactions and will inform strategies to improve cosolvent tolerance.

Tan, L., et al. 2023. "Amphiphilic Co-Solvents Modulate Structure of Membrane Domains," *ACS Sustanable Chemistry & Engingeering.* DOI:10.1021/acssuschemeng.2c06876.

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Characterization, Neutron Scattering, and Molecular Dynamic Simulation of the Lignin Carbohydrate Complex Structure and its Disruption

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https://sfa-biofuels.ornl.gov/

Project Goals: Recent work aimed at improving the conversion of biomass to advanced biofuels and bioproducts has highlighted the critical importance of solvent effects. These effects are important both in the efficient solvent-based deconstruction of biomass and in the product titer limitations of fermentations due to solvent-based destabilization of microbial membranes. This Science Focus Area provides fundamental knowledge about how solvents alter the structures of plant cell walls and microbial membranes. The overarching hypothesis is that knowledge of partitioning or binding of the solvent from the bulk phase to biomass or biomembranes can help predict maximal or minimal disruption. Solvents disrupt biological structures comprising amphiphilic molecules and polymers (e.g., membranes and biomass). Determining common biophysical principles of solvent disruption will lead to new understandings of how solvents affect the relevant structures. This information will help determine the ultimate microbial limits in tolerating specific solvents, as well as the eventual design of cosolvents best suited for pretreatment. Researchers integrate the power of world-class neutron scattering capabilities and leadership-class supercomputing facilities available at Oak Ridge National Laboratory (ORNL). These capabilities are complemented by expertise in biodeuteration and biomembranes at ORNL, plant cell wall chemistry at the University of Tennessee, and neutron scattering and membrane biophysics at the University of Cincinnati.

Effective conversion of biomass remains challenging. The three major components—cellulose, hemicellulose, and lignin—form a recalcitrant lignin–carbohydrate complex (LCC) that must be fractionated for valorization. The team's studies on molecular structural changes underline two approaches to improve biomass conversion: pretreatment improvement and feedstock genetic engineering. In the first case, researchers describe the mechanism of action of Cyrene in solubilization and fractionation of lignin during thermochemical pretreatment. In the second case, researchers investigated LLCs in pectin knockdown transgenic switchgrass and model composites to gain insight into the molecular details of lignin–carbohydrate interactions. Overall, this comprehensive analysis furthers understanding of the solvent effect during biomass fractionation and critical polymer interactions in plant cell walls that impact biomass recalcitrance.

Cyrene is the trademark name of dihydrolevoglucosenone, a biodegradable, non-toxic green dipolar aprotic solvent produced by the Circa Group on a scale of 50 tons per year. Cyrene effectively extracts a significant amount of lignin from hardwood, herbaceous species, and even softwood at an aqueous acidic mild temperature of 120°C (Wang et al. 2023). Nuclear magnetic resonance (NMR) revealed that the structure of extracted lignin was modified, correlating to the composition of the Cyrene cosolvent system.

Interactions between Cyrene and lignin were studied by molecular dynamic simulation (MD), revealing that Cyrene facilitated lignin solubilization and disrupted lignin aggregation. Cyrene also modified the cellulose fraction of biomass. Small-angle X-ray scattering (SAXS) showed no lignin aggregation on the surface of microfibrils after pretreatment. However, small-angle neutron scattering (SANS) showed that the distances between cellulose microfibrils increased

after Cyrene pretreatment and then decreased to a level similar to untreated biomass after incubation with a dilute alkaline solution. This suggests that the presence of Cyrene between microfibrils and its removal after alkaline incubation. This comprehensive analysis demonstrated the high potential of Cyrene cosolvent fractionation in extracting lignin and enhancing fermentable sugar yield by revealing the molecular interactions between Cyrene and LCCs.

Engineered plants with reduced pectin exhibit lower recalcitrance towards conversion to biofuels (Biswal et al. 2018), but complexes of pectin and lignin have not been confirmed as playing a role in recalcitrance. Researchers utilized a model composite system to investigate the effects of pectins on lignin polymerization (Shah et al. 2023). The lignin monomer coniferyl alcohol, protiated or deuterated, was polymerized *in vitro* by the hydrogen peroxide–horseradish peroxidase method in the presence of homogalacturonan, a linear pectin found in grasses. These composites were characterized by Fourier-transform infrared spectroscopy, solid-state NMR, SAXS, and SANS experiments. The lignin-pectin composites were compared to lignin synthesized without pectin and a physical mixture of pectin and lignin. Lignin particle sizes were smaller in the composites, and interconnected networks were formed. A unique ester bond was detected, supporting the existence of covalent bonds as well as hydrophobic interactions between lignin and pectin. These insights into the role of pectin in lignin deposition in the cell wall may inform improvements to biomass and its deconstruction for biofuels and bioproducts.

Biswal, A. K, et al. 2018. "Sugar Release and Growth of Biofuel Crops are Improved by Downregulation of Pectin Biosynthesis," *Nature Biotechnology* **36**(3), 249–57. DOI:10.1038/nbt.4067.

Shah, R. S., et al. 2023. "Evidence for Lignin–Carbohydrate Complexes from Studies of Transgenic Switchgrass and a Model Lignin–Pectin Composite," *ACS Sustainable Chemistry & Engineering* **11**(3),15941–50. DOI:10.1021/acssuschemeng.3c04322.

Wang, Y.-Y., et al. 2023. "Characterization and Molecular Simulation of Lignin in Cyrene Pretreatment of Switchgrass," *Green Chemistry*. DOI:10.1039/D3GC02239K2.

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Science Focus Area
µBiospheres: Community Systems Biology of Microscale Interactions for Sustainable Bioenergy

Mechanisms and Flux Measurements of Microbial Processing of Photosynthetically Fixed Algal Carbon Using Isotope Tracing and Secondary Ion Mass Spectrometry

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https://bio-sfa.llnl.gov/

Project Goals: Algal and plant systems have the unrivaled advantage of converting solar energy and carbon dioxide (CO_2) into useful organic molecules. Their growth and efficiency are largely shaped by the microbial communities in and around them. The µBiospheres Science Focus Area seeks to understand phototroph–heterotroph interactions that shape productivity, robustness, the balance of resource fluxes, and the functionality of the surrounding microbiome. Researchers hypothesize that different microbial associates not only have differential effects on host productivity but can change an entire system's resource economy. This approach encompasses single-cell analyses, quantitative isotope tracing of elemental exchanges, omics measurements, and multiscale modeling to characterize microscale impacts on system-scale processes. Researchers aim to uncover crosscutting principles that regulate these interactions and their resource allocation consequences to develop a general predictive framework for system-level impacts of microbial partnerships.

Photosynthetic carbon (C) fixation by algae and cyanobacteria represents half the global C fixation on Earth and holds promise as a strategy for nonfossil fuel–based generation of biofuels. Loss of fixed C as dissolved organic matter (DOM) through exudation, lysis, and as viral progeny after infection represent critical fluxes that become a source of biomass for the algal microbiome. Using four different experimental systems, researchers used stable isotope tracing with ^{13}C and nitrogen-15 (^{15}N) labeling combined with single-cell resolution isotope analysis by nanoscale secondary ion mass spectrometry to investigate mechanisms and fluxes of algal organic C into the algal microbiome.

The first two experimental systems examine the model biofuel-producing diatom *Phaeodactylum tricornutum* and bacterial isolates that grow using *P. tricornutum*–fixed C. Using a porous microplate that cocultivates bacteia near a constant source of algal dissolved organic matter, the team tested whether the isolates compete for it. Researchers found that the presence of some bacterial strains inhibited uptake of algal-derived C by *Marinobacter*, a common algal-associated bacterium, while others did not. This suggests that niche partitioning and competition directly influence C from algae to bacteria.

In the second experimental system, researchers are examining how oxidative stress, which is prevalent in high-biomass and high-light ecosystems, impacts the transfer of algal C and N into heterotrophic bacteria. The team aimed to compare bacteria that relieve oxidative stress versus those that do not, and thus used two photoheterotrophic bacteria (one mutualistic and the other commensal under oxidative stress). Researchers quantified the transfer of algal C and N into the bacteria with or without the addition of hydrogen peroxide. They found that the non-mutualist incorporated more C and N under oxidative stress, whereas the mutualist did not change its uptake.

The third experimental system used the alga *Chlamydomonas reinhardtii* and the vitamin-producing soil bacterium *Mesorhizobium japonicum*, previously used as a model for vitamin exchange for fixed C. The team unexpectedly found little algal-derived C exchanged from alga to bacterium. The modest amounts could be explained by a low level of algal cell lysis, suggesting this bacterium grows using algal lysate rather than exudate.

For the fourth experimental system, the team aimed to examine the flux of algal-derived C into bacteria using algal viruses as a mechanism. Researchers isotope-labeled a giant virus infecting the alga *Emiliania huxleyii* and added this isotope-labeled viral fraction to a complex aquatic microbial community. Viral particles decreased faster in the presence of the microbial community than without cells. This

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corresponded to increased isotope labeling into bacteria and eukaryotic protist cells, demonstrating another mechanism for algal-derived C feeding the microbial loop.

Direct isotope measurements in these four experimental algal systems demonstrated the capability to measure fluxes from algae to bacteria. The team found they are impacted by mechanisms (e.g., cell lysis, exudation, and viral particles) and environmental factors (e.g., oxidative stress and competition by other bacteria).

Funding Statement: Part of this work was performed under the auspices of DOE at Lawrence Livermore National Laboratory (LLNL) under contract number DE-AC52-07NA27344, and supported by the BER Genomic Science Program under the LLNL μBiospheres SFA, FWP SCW1039.

Beneficial Plant–Fungal Partnerships in the Resource Economy of Bioenergy Grasses

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Project Goals: Algal and plant systems have the unrivaled advantage of converting solar energy and carbon dioxide (CO_2) into useful organic molecules. Their growth and efficiency are largely shaped by the microbial communities in and around them. The μBiospheres Science Focus Area seeks to understand phototroph–heterotroph interactions that shape productivity, robustness, the balance of resource fluxes, and the functionality of the surrounding microbiome. Researchers hypothesize that different microbial associates not only have differential effects on host productivity but can change an entire system's resource economy. This approach encompasses single-cell analyses, quantitative isotope tracing of elemental exchanges, omics measurements, and multiscale modeling to characterize microscale impacts on system-scale processes. Researchers aim to uncover crosscutting principles that regulate these interactions and their resource allocation consequences to develop a general predictive framework for system-level impacts of microbial partnerships.

Multipartite mutualisms between plants and microbiota can enhance plant productivity, stress resilience, and carbon (C) allocation belowground. Researchers are investigating context-dependent mutualisms between *Panicum virgatum* (switchgrass, a cellulosic bioenergy grass), Panicum hallii (a model for bioenergy grasses), and mycorrhizal and endophytic fungi. The team is interested in how C flows are mediated by plant-associated fungi and altered by environmental stress (e.g., drought). In return, it is thought that hyphosphere microbes surrounding fungal hyphae enable root-associated fungi to obtain resources (nitrogen, phosophorus, and water) that they provide to their hosts, but the mechanisms that enable this crosskingdom cooperation are unknown. Researchers are investigating these questions using (1) $^{13}CO_2$ stable isotope probing (SIP) and metabolomics, and (2) live imaging and spatial metabolomics coupled to metabolic modeling.

Fungal root endophytes can alleviate plant drought stress, but their effects on soil microbial activity and C flows during drought are poorly understood. The team used $^{13}CO_2$ labeling chambers, root exclusion cores, quantitative SIP (qSIP), and metabolomics to investigate how two functionally distinct root endophytes influenced rhizosphere and hyphosphere C dynamics in moisture-limited soils planted with *P. hallii*. Researchers compared the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* with the *S*ebacinales endophytic fungus *Serendipita bescii*. CO_2 efflux and $^{13}CO_2$ efflux were greater from fungal-inoculated versus uninoculated soils, indicating that these fungi facilitated faster turnover of both native soil organic matter and ^{13}C photosynthates. However, the team did not measure a net reduction in total soil C. Microbial ^{13}C assimilation was greater in fungal-inoculated soil, and a distinct microbial consortia assimilated ^{13}C in each treatment. The hyphosphere exometabolome was primarily structured by time and was distinct between well-watered and drought conditions; a subset of metabolites differed by the specific fungal partner inoculant. These results provide a putative mechanism to explain the previous observation that fungal root endophytes help maintain bacterial growth potential, growth efficiency, and diversity following moisture limitation (Hestrin et al. 2022).

Fungal exudates are a key form of C in the hyphosphere and may mediate a metabolomic conversation between fungi and their microbiome. To relate fungal network development and exudation to microbiome nutrient acquisition, researchers are coupling spatially resolved analyses to a metabolic modeling simulation platform called "Toadstool." These experiments start with automated live imaging and network identification to generate baseline structural data for the platform. Toadstool is built on a stochastic network

representation of *R. irregularis* growth and resource allocation in soil, coupled to differential equations that represent light- and nutrient-dependent growth of *P. virgatum*. Currently, researchers are developing a method to spatially map AMF metabolites using matrix-assisted laser desorption and ionization (MALDI) that can pair with live imaging data. Toadstool was designed to interact within a diffusive and advective grid, allowing a direct interface with spatially resolved metabolic models of *R. irregularis* hyphae and their bacterial partners. This approach enables a comparison of predicted metabolite exchanges with MALDI metabolite imaging. The model is intended to predict feedbacks between growth, plant–AMF–bacterial community resource exchange, and the soil matrix. This work will shed light on how multipartite biological interactions impact the soil resource economy.

Hestrin, R., et al. 2022. "Plant-Associated Fungi Support Bacterial Resilience Following Water Limitation," *The ISME Journal* 1–11.

Funding Statement: This work was performed under the auspices of DOE at Lawrence Livermore National Laboratory (LLNL) under contract DE-AC52-07NA27344, and supported by the BER Genomic Science Program under the LLNL μBiospheres Science Focus Area, FWP SCW1039.

Iron-Mediated Microbial Interactions with Primary Producers in Terrestrial and Aquatic Systems

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https://bio-sfa.llnl.gov/

Project Goals: Algal and plant systems have the unrivaled advantage of converting solar energy and carbon dioxide (CO_2) into useful organic molecules. Their growth and efficiency are largely shaped by the microbial communities in and around them. The μBiospheres Science Focus Area seeks to understand phototroph–heterotroph interactions that shape productivity, robustness, the balance of resource fluxes, and the functionality of the surrounding microbiome. This team hypothesizes that different microbial associates not only have differential effects on host productivity but can change an entire system's resource economy. This approach encompasses single-cell analyses, quantitative isotope tracing of elemental exchanges, omics measurements, and multiscale modeling to characterize microscale impacts on system-scale processes. Researchers aim to uncover crosscutting principles that regulate these interactions and their resource allocation consequences to develop a general predictive framework for system-level impacts of microbial partnerships.

Iron (Fe) is an essential micronutrient, and microbial Fe acquisition strategies are predictive of host health across an array of environments. Despite this, a molecular-level understanding of microbial Fe acquisition influence on partnership outcomes and system-level carbon (C) flux is lacking. Here, the group examines both algal–bacterial and plant–fungal interactions in response to changes in Fe to elucidate exchange mechanisms governing these interactions.

Researchers first examined bacterial isolates that grow with the model diatom *Phaeodactylum tricornutum*. With the Boiteau laboratory at the University of Minnesota, the team grew *P. tricornutum* alone or in coculture with isolates under different concentrations of Fe dust. The group used global exometabolomic profiling to determine the diatom Fe limitation response and compounds consumed or exuded by bacteria. When *P. tricornutum* growth is limited by Fe, the algae decreases production of saturated fatty acids relative to Fe-replete conditions. Some bacteria aid in algal Fe acquisition under limitation, significantly increasing algal abundance ($p<0.02$). Other bacteria compete for scarce Fe resources, significantly inhibiting algal growth under low Fe dust ($p<0.05$). These shifts in algal growth with and without bacterial partners under low Fe were accompanied by metabolomic shifts, providing insight into molecular drivers of growth.

To understand the systems-level impacts of these Fe acquisition strategies, it is important to also examine the role of bacteria–bacteria interactions. Researchers therefore examined effects of Fe limitation in a simplified community (~30 bacterial taxa) grown with *P. tricornutum* as the sole organic C source. The group found that under conditions of low dissolved Fe, the bacteria:alga ratio decreases significantly and community composition shifts. The team hypothesizes that Fe limitation enriches for taxa that can grow under reduced Fe (e.g., siderophore secretion) or low organic C. To determine whether algal organic C quality, as well as Fe-chelating compounds, shift with Fe, the group profiled exometabolites. Initial results suggest algal-bacterial exudation and consumption are distinct under different Fe regimes. This points to a tight coupling of Fe and C in the *P. tricornutum* microbiome and regulation by external factors (e.g., nutrient limitation) and host physiological state.

On the plant-fungal side, with the Hawkes laboratory at North Carolina State University, researchers isolated diverse Ascomycota fungi from switchgrass roots and grew plants with single fungal partners. The team found significant fungus-dependent variation in plant Fe content, suggesting the different fungi had distinct Fe acquisition strategies or host transfer mechanisms. To test this, the Boiteau laboratory conducted global metabolomic profiling of 19 fungal

isolates under Fe limitation. The non-siderophore producer *Trichoderma* had the largest negative effect on plant Fe, while the fusarinine-producing strain *Chaetomium* resulted in the largest increase in plant root Fe uptake (1.8 and 2.4-fold in shoots and roots, respectively, relative to uninoculated controls). Fungi with distinct Fe uptake and metabolic strategies differentially impact plant Fe acquisition and are likely to constrain switchgrass productivity.

In summary, the group finds that Fe has an important role in phototroph–host interactions and system productivity. By characterizing microbial Fe acquisition strategies and associated C flux, researchers aim to gain a predictive understanding of the role of Fe across a broad range of host–microbe interactions.

Funding Statement: This work was performed under the auspices of DOE at Lawrence Livermore National Laboratory (LLNL) under contract DE-AC52- 07NA27344, and supported by the BER GSP under the LLNL μBiospheres Science Focus Area, FWP SCW1039 LLNL-ABS-860548.

The Lawrence Livermore National Laboratory Cryo-NanoSIMS: the Next Generation for High Spatial Resolution Functional Analysis

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https://bio-sfa.llnl.gov/

Project Goals: Algal and plant systems have the unrivaled advantage of converting solar energy and carbon dioxide (CO_2) into useful organic molecules. Their growth and efficiency are largely shaped by the microbial communities in and around them. The μBiospheres Science Focus Area seeks to understand phototroph–heterotroph interactions that shape productivity, robustness, the balance of resource fluxes, and the functionality of the surrounding microbiome. Researchers hypothesize that different microbial associates not only have differential effects on host productivity but can change an entire system's resource economy. This team's approach encompasses single-cell analyses, quantitative isotope tracing of elemental exchanges, omics measurements, and multiscale modeling to characterize microscale impacts on system-scale processes. The team aims to uncover crosscutting principles that regulate these interactions and their resource allocation consequences to develop a general predictive framework for system-level impacts of microbial partnerships.

In 2002, Lawrence Livermore National Laboratory (LLNL) installed a nanoscale secondary ion mass spectrometry (SIMS) 50 for the study of microbial ecology. NanoSIMS was unproven technology at the time, but it was rapidly adopted for microbial ecology because of its ability to trace isotopically labeled substrates into microbial communities. One limitation, however, has been that samples must be prepared for high vacuum, which results in the loss of soluble species and can cause significant sample alteration. To overcome this limitation, LLNL worked with the manufacturer, CAMECA Instruments, to develop the next-generation NanoSIMS with a cryogenic stage. This winter at LLNL, CAMECA began installing the prototype product of that collaboration: a first-of-its-kind cryo-NanoSIMS. This unique instrument has an analysis stage that can be cooled to liquid nitrogen temperatures and a cryogenic system for sample handling and introduction. With this cryogenic capability, researchers will be able to analyze frozen hydrated samples, which will capture soluble molecules that were previously lost during room temperature sample preparation methods. Sample freezing can also maintain spatial relationships among organisms without the addition of embedding resins that remove soluble molecules and mask the organic molecules researchers seek to detect.

Another remarkable feature of the new cryo-NanoSIMS is its new 10 nm cesium ion source, which is used to image and analyze samples. The cesium ion source generates an ion beam with unprecedented intensity, allowing the LLNL prototype cryo-NanoSIMS to achieve 10 nm spatial resolution, and enabling researchers to resolve structures down to the size of a single phage.

In addition, the prototype cryo-NanoSIMS has been upgraded to improve useability and throughput, including redesigned electronics, sample stage, optical imaging, and sample introduction systems. The new electronics allow low-energy sample analysis, improving the ability to resolve small structures by increasing depth resolution. The stage now has optical encoding and achieves better than 500 nm reproducibility, allowing 2 to 100 times faster automated analysis of selected targets, depending on the target size and analysis duration. The optical imaging system can now resolve micron-scale features and be used for automated sample registration, easing navigation. An automated sample introduction further increases ease of use and productivity.

The team will use its cryo-NanoSIMS for a wide range of applications, including host–microbe interactions in bioenergy-relevant systems and soil carbon cycling. Researchers will present performance data, including initial analyses of microbial associations in perennial grasses and microalgae.

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Science Focus Area
Quantitative Plant Science Initiative (QPSI)

Quantitative Plant Science Initiative: Integrating Functional Genomics with Biomolecular-Level Experimentation to Understand Adaptation to Micronutrient Stress in Poplar and Sorghum

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https://genomicscience.energy.gov/bnlqpsi/

Project Goals: The Quantitative Plant Science Initiative (QPSI) is a capability that aims to bridge the knowledge gap between genes and their functions. A central strategy is combining genome-wide experimentation and comparative genomics with molecular-level experimentation. In this way, the project team leverages the scalability of omics data and bioinformatic approaches to capture system-level information, while generating sequence-specific understanding of gene and protein function. Incorporating molecular-level experimentation in the workflow addresses the question of how proteins function and establishes mechanistic insight into how sequence variation impacts phenotype. This knowledge serves as a touchstone for accurate genome-based computational propagation across sequenced genomes and forms a foundation for robust predictive modeling of plant productivity in diverse environments.

To understand how the bioenergy crops poplar and sorghum respond to metal bioavailability, with a view toward improving bioenergy crop resilience, the research team performed integrated, large-scale, multi-genotype omics experiments, computational simulation, and gene/protein-focused molecular-level experimentation. The project has two objectives. Objective 1 is to determine the genome-wide responses to zinc (Zn) and iron (Fe) availability in sorghum and poplar and identify the major genes involved in leaf-level acclimation to metal ions. The team performed time-series and genotype-specific multiomics experiments and obtained datasets useful for the identification of key functional genes.

Objective 2 is to identify the molecular-level functions of key proteins and validate them by overexpression and loss-of-function phenotyping. Following the team's recent discovery of previously unknown Zn chaperones in eukaryotes (Pasquini et al. 2022), a structure–function study of these novel proteins was completed and a plant-specific Zn-homeostatic mechanism that involves intracellular Zn transferases was identified (Zhang et al. 2023). The team also discovered a new heme sensor involved in cofactor-dependent post-translational regulation at the intersection of photosynthesis and respiration (Grosjean et al. 2024). The structure of a Zn transporter dimer was determined, revealing a flexible loop for sensing cellular Zn content and regulating Zn uptake from the environment (Pang et al. 2023).

In addition to molecular-level discoveries in micronutrient homeostasis, a protoplast-based experimental system was used to discover a key gene regulatory network that controls sorghum flowering time and biomass production (Tadesse et al. 2024). While working with Zn and Fe micronutrient stresses in the current project phase, there will be subsequent opportunities to incorporate other real-world conditions through the addition of field experiments, which address the impacts of soil geochemistry, microbiome, and rhizosphere. Additional opportunities include studying bioenergy crops and environmental interactions.

* Presenting Author

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Energy and Environmental Research Center (EERC)

Terraforming Soil Energy Earthshot Research Center: Accelerating Soil-Based Carbon Drawdown Through Advanced Genomics and Geochemistry

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Project Goals: The Terraforming Soil Energy Earthshot Research Center (EERC) will study biological and geological solutions to accelerate scalable, affordable carbon drawdown in the United States' 166 million hectares of agricultural soils. Research objectives include gene-edited plants and microorganisms that accelerate carbon sequestration, strategies that encourage soil mineral–organic interactions, and models that predict carbon durability in small soil pores, as well as regional-scale estimates of locations with opportunities for increased soil carbon removal.

To reduce the United States' net carbon dioxide (CO_2) emissions to zero and limit the impacts of global warming, it is essential to actively remove CO_2 from the atmosphere. Soils store a vast amount of carbon (C) in organic and inorganic forms, on the order of 3,000 gigatons globally. This is more carbon than is found in the atmosphere and on land combined. While U.S. agricultural soils have lost a vast amount of carbon in the past century due to cultivation and erosion, there is clear potential to reverse this trend and actively manage agricultural lands with strategies that capture CO_2 from the atmosphere. The Terraforming Soil EERC will research new bio- and geoengineered techniques to understand, predict, and accelerate scalable and affordable CO_2 drawdown in soils. The Center's overarching goal is to advance fundamental understanding of CO_2 drawdown in soils through both organic and inorganic pathways by measuring soil C storage capacity, durability, and regional variations that have bearing on land management practices. In Objective 1, synthetic biology tools will be used to accelerate naturally occurring plant and microbial traits that shape CO_2 fixation processes, organic matter formation, and mineral dissolution. Combined genome sequencing and isotope tracing approaches will be used to quantify the fundamental mechanisms of how organic matter accrues over time and which traits of plants

* Presenting Author

and microorganisms need to be better reflected in process models. In Objective 2, the Center will focus on positive interactions that can occur during the weathering of primary minerals and the formation of organic matter–mineral complexes. Together, these have dramatic potential to accelerate soil CO_2 drawdown via combined organic and inorganic pathways. But currently, interactions between soil weathering, soil biology, and organic matter cycling are poorly understood. The Center's field and laboratory-based studies will measure how soil management approaches can be stacked together to optimize total CO_2 drawdown via co-deployment of novel engineered crops or microbes, silicate minerals, or organic amendments. Research for Objective 3 will integrate new modeling capabilities and data exploration to enable better predictions of soil CO_2 drawdown in both space and time. Novel micro- and macro-scale simulation tools will be combined with advanced modeling, machine learning, and data science approaches, allowing the Center to better forecast the potential impacts of new soil CO_2 drawdown approaches at multiple scales. The Terraforming Soil EERC team includes world-class experts in soil carbon cycling, photosynthesis biochemistry, plant and microbial gene engineering and genomics, mineral geochemistry, machine learning, exascale modeling and computing, additive manufacturing, and *in situ* isotope-based characterization. Throughout the research program, the Center will bridge cutting-edge analytical and computational studies with a commitment to engage with community stakeholders by exploring the technical, social, and economic implications of engineered soil CO_2 drawdown. The Center will emphasize diverse training opportunities for students and early career scientists and amplify equity and inclusion throughout the research pipeline.

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Interactions Between Enhanced Rock Weathering and Soil Organic Carbon Cycling in Coordinated, National-Scale Field Trials

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Project Goals: The Terraforming Soil Energy Earthshot Research Center (EERC) will study biological and geological solutions to accelerate scalable, affordable carbon (C) drawdown in the United States' 166 million hectares of agricultural soils. Research objectives include gene-edited plants and microorganisms that accelerate C sequestration, strategies that encourage soil mineral–organic interactions, and models that predict carbon durability in small soil pores, as well as regional-scale estimates of locations with opportunities for increased soil carbon removal.

If applied at scale on croplands, enhanced rock weathering (ERW) could feasibly remove 0.5 to 2 petagrams of C each year through amendments of relatively fast-reacting crushed alkaline minerals (e.g., crushed basalt rock). However, several critical unknowns limit the scalability of ERW as a C drawdown strategy. First, published data remain limited and existing field studies employ different approaches for measuring inorganic C removal, making it difficult to compare the net C removal of ERW across agricultural regions. Second, the impacts of ERW on soil organic carbon (SOC) and soil microbial communities are poorly understood. Given the massive size of the SOC reservoir, small SOC gains or losses could either amplify or entirely negate the inorganic C drawdown benefits of ERW. The EERC will address this gap by assessing impacts of field trials across different major agricultural regions within the United States (California, the Midwest, and Southeast) using a standardized set of total C drawdown measurements. Here, the team presents some preliminary data from existing field trials.

At three field trials in the Central Valley of California, the team found that stocks of mineral-associated organic matter (MAOM) were 8 to 16% lower in the surface soil (0 to 10 cm) of plots with crushed basalt amendments versus unamended controls. At the sites where baseline data was available, crushed rock amendments did not lead to net losses of total soil organic matter relative to

* Presenting Author

initial conditions; however, the accrual rate of surface soil MAOM over the two-year period was 60 to 97% lower in plots with crushed rock relative to control plots. At the field trial in Minnesota on a typical corn–soybean rotational field, researchers found increased porewater alkalinity and increased yield in acidic soils treated with steel slag, though minimal effect of basalt on inorganic C removal, SOC, or plant yield. At the Illinois site, after 3 years of basalt application, the group found a signal for elevated or negligible shifts in crop yields, with the largest shifts observed in oats and soy. Using a cation accounting method, which tracks calcium and magnesium concentrations in the silicate phases relative to detrital elements, researchers found evidence of significant amounts of basalt weathering (representing multiple tons of carbon dioxide removal per hectare per year). Despite high basalt application rates and a signal for extensive weathering, the soil pH in the plots stayed near neutral, suggesting limited agronomic risk from the practice in these soils.

In sum, this group found evidence of inorganic C removal at some sites and contrasting effects on SOC at the sites where it was measured. The next phase of field trials will be to measure the effects of ERW on inorganic C and SOC using a similar experimental design, a standardized sampling protocol, and a common set of C measurements in order to facilitate more accurate and comparable estimates of net C removal across regions. Researchers will also investigate how co-applying organic amendments with crushed rock may hold promise for optimizing microbial-mediated inorganic and organic C removal.

Funding Statement: This research is based upon work of the Lawrence Livermore National Laboratory (LLNL) Terraforming Soil EERC, supported by the DOE Office of Science BER program, Basic Energy Sciences (BES) program, and Advanced Scientific Computing Research (ASCR) program under award number SCW1841 to LLNL, and subcontracts to University of California–Davis; Yale University; Carleton College; and Lawrence Berkeley National Laboratory. Work at LLNL was performed under DOE contract DE-AC52-07NA27344. Initial work at the Working Lands Innovation sites was supported by California Strategic Growth Council grant CCR20007. Field trials in Minnesota are supported by the National Science Foundation (NSF-EAR 2208133).

RESTOR-C: Center for Restoration of Soil Carbon by Precision Biological Strategies

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Project Goals: The goal of the Center for Restoration of Soil Carbon by Precision Biological Strategies (RESTOR-C) is to harness plants and microbes to increase carbon flux into soil carbon storage pools to form persistent carbon that is stable for >100 years. This will address the Carbon Negative Shot goal to remove carbon dioxide (CO_2) from the atmosphere and durably store it at meaningful scales for less than $100 per net metric ton of CO_2-equivalent within a decade.

Soil carbon represents a vast global carbon reservoir that has become depleted through human activities. Hence, soil carbon restoration can be used to sequester carbon at massive scales while improving soil fertility. To exploit this natural carbon sink and advance toward the cost and scale goals of the DOE Carbon Negative Shot, RESTOR-C will develop plant- and microbe-based strategies to increase accumulation of persistent carbon in soil. These strategies are designed to increase the amount of atmospheric carbon fixed by plants and increase the amount of the fixed carbon that is channeled belowground as soil-persistent carbon. To accomplish this goal, the Center will apply cutting-edge molecular and computational methods to overcome key obstacles to persistent carbon storage in four key domains. The project's Soil Division will explore the chemical, biological, and environmental factors that govern the persistence of carbon in soils to enable the development of stable, long-term carbon storage solutions with a focus on arid and marginal lands. This work will combine soil carbon dating, advanced metabolomics methods, and artificial intelligence to determine the nature of the oldest carbon and features that influence its persistence. The Plant Division will design plant genotypes that efficiently capture and sequester carbon through a combination of increased photosynthetic efficiency and optimized root phenotypes. These efforts will focus on sorghum, a stress-tolerant C_4 bioenergy crop that can grow in a range of soils and climates with minimal nutrient inputs, building on team members' experience

engineering improved photosynthetic efficiency and altered root phenotypes in plants. The Microbial Division will identify and optimize microbial communities to promote carbon retention in soil. Methods to achieve this include chemical analysis to identify microbes that produce persistent carbon, omics-based analyses to determine microbial niche preferences, enrichment and selection methods to obtain carbon-storage-promoting microbes, and artificial intelligence–guided high-throughput experiments to test and improve microbial strategies for soil carbon deposition. Finally, the Scaling and Impact Division will model, predict, evaluate, and optimize cost and scale of soil carbon sequestration approaches. This work will build and connect field-scale reactive transport and agroecosystem-scale models of carbon dynamics with national-scale models of economic feasibility to predict the impacts of carbon sequestration approaches, evaluate implementation strategies, and test promising approaches at the field level.

This research will break new ground in multidisciplinary research, leveraging unique expertise at two national laboratories and four university partners, including two minority-serving institutions, to integrate recent developments and breakthroughs spanning the biological, ecological, chemical, and computing sciences. At the end of the 4-year period, the Center will have validated plant–microbe strategies to increase carbon at target field sites in California and New Mexico, as well as a dramatically expanded knowledgebase and set of capabilities to rapidly extend these approaches to other locations and crops. In the long term, these methods have the potential to restore carbon in U.S. agricultural lands, forging the way toward a carbon negative future.

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Vision-Driven RhizoNet: Foundations for Systematic Measurement of Plant Root Biomass

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Project Goals: The goal of Center for Restoration of Soil Carbon by Precision Biological Strategies (RESTOR-C) is to harness plants and microbes to increase carbon flux into soil carbon storage pools to form persistent carbon that is stable for >100 years. This will address the Carbon Negative Shot goal to remove carbon dioxide (CO_2) from the atmosphere and durably store it at meaningful scales for less than $100 per net metric ton of CO2-equivalent within a decade.

Improving root traits is an important research area for soil carbon sequestration. To accelerate this research, researchers have developed the EcoBOT, an innovative robotic system designed for plant imaging including growth and monitoring of plants in specialized devices called EcoFABs that enable detailed root scans. The EcoBOT allied to EcoFABs can generate many hundreds of root scans each week; so, automated computer vision tools based on machine learning are needed to rapidly process the data. To address this challenge, this group is building RhizoNet, a deep learning–based workflow tailored for precise semantic segmentation of plant root imagery, including modules for analysis and measurements of root biomass growth. RhizoNet overcomes many challenges by employing a sophisticated Residual U-Net architecture that significantly improves prediction accuracy. This is complemented by a convex hull operation aimed at precisely delineating the primary root component over time, thus facilitating a more accurate assessment of root biomass and its growth. Its robust root detection model has demonstrated generalization capabilities across a wide range of experimental conditions, underscoring its utility in standardizing and objectifying the analysis of thousands of root images. By integrating RhizoNet into EcoBOT's operational framework, the process of acquiring and analyzing root scans can be significantly streamlined, reducing the need for manual intervention and thereby increasing throughput and accuracy in root growth studies. This automation is crucial for real-time monitoring and autonomous decision-making. Furthermore, the application of RhizoNet to plant root analysis highlights the broader implications of semantic segmentation technology fields to optimize plant growth, enhance crop yields, and contribute to sustainable agricultural practices.

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Early Career

Understanding Plant/Environmental Interactions Using Single-Cell Approaches

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Biomass derived from plant feedstocks is a renewable and sustainable energy resource, but these resources are vulnerable to environmental stress such as water and nutrient limitations. Understanding how cells work independently and in concert to regulate plant responses to their environment, including their surrounding microbial community and abiotic stress, will be crucial to improving their performance. This Early Career Research Project applies several cutting-edge, single-cell, and spatially resolved transcriptome sequencing approaches to construct a comprehensive single-cell resource for plants and to better understand the complexity behind environmental responses among diverse cell types. To this end, researchers have profiled thousands of individual sorghum root cells grown under normal and phosphate-limited conditions. The team has also begun to profile *Brachypodium* root and leaf cells using single-nuclei RNA sequencing. Researchers are currently integrating this nascent data with additional single-cell data from other species, including maize. The project is also characterizing environmental stress using other advanced profiling methods, including spatial transcriptomics and spatial metabolomics, on plant–arbuscular mycorrhizae interactions. The team hopes to build a multispecies model of cell type–specific environmental responses.

Understanding the Effects of *Populus*–Mycorrhizal Associations on Plant Productivity and Resistance to Abiotic Stress

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Project Goals: The overarching goal of this project is to create sustainable, managed ecosystems where important biofeedstocks can be produced while simultaneously maximizing soil health and mitigating adverse impacts of climatic change.

Over the past 2 decades, it has become clear that symbiotic host–microbe interactions alter the way plants grow and respond to abiotic and biotic stress. Harnessing diversity within these plant–microbe associations provides an opportunity to create sustainable, multipurpose ecosystems. Within these managed ecosystems, science can produce energy necessary to meet global needs while maximizing soil health and mitigating adverse impacts to climate. Therefore, to increase sustainability within DOE-relevant biofeedstocks, researchers aim to develop plant–microbial pairings tailored for specific environmental conditions. First, researchers are identifying genetic variation within plant hosts to select plants that are tolerant to abiotic stress. Next, scientists are complementing these plants with varied belowground microbial partners to alter plant performance and ecosystem carbon cycling.

First, this research group identified *Populus* genotypes that varied in their responses to drought without associated microbial partners. Over the last 2 years, team members conducted a series of greenhouse experiments where they identified variation in *Populus* response to drought using both hyperspectral imaging and assessing changes in plant physiology across 39 genotypes of *P. trichocarpa*, 39 of *P. deltoides,* and 26 hybrid genotypes (*P. trichocarpa* x *P. deltoides*). Overall, researchers found differences in plant phenotypes across genotypes and in response to drought. Interestingly, drought-tolerant genotypes maintained higher levels of stomatal conductance

* Presenting Author

during drought relative to drought-susceptible genotypes. Genotypes in these experiments will be leveraged in manipulative studies with mycorrhizal isolates to examine if mycorrhizae can increase host stress tolerance in both drought-susceptible and -tolerant genotypes.

Next, this team characterized variation in mycorrhizal community dynamics across drought-tolerant and -susceptible *P. trichocarpa* genotypes planted in a common garden in Davis, Calif. In February and July 2022, researchers collected root and rhizosphere samples from drought-tolerant and -susceptible *P. trichocarpa*. Across these genotypes, the team found both arbuscular mycorrhizal and ectomycorrhizal (ECM) fungi colonized the roots, and drought-tolerant genotypes had a greater percentage of hyphae, greater number of arbuscules, and a larger Hartig net compared to drought-susceptible trees. Researchers found that mycorrhizal diversity and community composition varied between well-watered and drought treatments and across plant genotypes. Fungal isolations yielded potentially new ECM taxa that can be leveraged in future experiments.

Finally, in the fall of 2023, team members collected root and rhizosphere samples from *P. trichocarpa* and *P. deltoides* across natural precipitation gradients in Washington and Texas, respectively. Within these collections, researchers are characterizing variation in mycorrhizal community composition, colonization, and abundance. Culture collections are being developed for future experimentation.

Combined, these initial efforts highlight significant genetic variation in the response of *Populus* to drought, and demonstrate variation in belowground mycorrhizal communities across drought-tolerant and -susceptible genotypes. Plant and fungal resources resulting from these experiments will be used to develop tailored plant–fungal partnerships to alter host abiotic stress tolerance and soil carbon cycling.

Funding Statement: This work was supported by the DOE Office of Science through the BER Early Career Research program. The *P. trichocarpa* genome-wide association study (GWAS) plantation in Davis, Calif., was developed and is maintained through the Center for Bioenergy Innovation, Bioenergy Research Center funded by BER.

Study of the 4-Hydroxybenzoate Catabolic Pathway in White-Rot Fungi via Biochemical and Structural Enzyme Characterization

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Project Goals: The overall goal of this Early Career Award project is to test the hypothesis that white-rot fungi (WRF) can simultaneously depolymerize lignin extracellularly and catabolize depolymerization products intracellularly as carbon (C) and energy sources. The results from this project will lead to improved understanding of lignin utilization by WRF in nature and enable identification of promising fungal strains and enzymes for lignin catabolism and valorization.

WRF are the most effective decomposers of lignin in nature. However, their ability to utilize aromatic compounds derived from lignin as sources of carbon was only recently demonstrated (del Cerro et al. 2021). That study proposed that two WRF species (*Trametes versicolor* and *Gelatoporia subvermipora*) employ the hydroxyquinol pathway to catabolize certain lignin-related aromatic compounds, such as 4-hydroxybenzoate. Although this pathway has not been extensively studied compared to the β-ketoadipate pathway, it represents an alternative route for incorporating aromatic compounds into central C metabolism in a subset of microorganisms. Due to the limited availability of genetic tools for studying WRF, understanding of gene–function relationships in these fungi is hindered. Therefore, researchers heavily rely on enzymatic assays to investigate biochemical conversions and validate complete catabolic pathways. This study conducted biochemical analyses of seven enzymes derived from two WRF species and validated a four-enzyme conversion pathway from 4-hydroxybenzoate to β-ketoadipate, a catabolic intermediate that enters central C metabolism. Additionally, researchers have solved the crystal structure of four of these enzymes and have analyzed their catalytic activity and structural features in comparison to homologous enzymes found in bacteria. Overall, this research significantly advances the understanding of the intracellular pathways

involved in the breakdown of aromatic compounds within WRF and pinpoints key mechanistic disparities between fungal and bacterial systems. These insights are particularly valuable for the development of enzymatic or microbial biocatalysts aimed at producing high-value chemicals from aromatic compounds, whether in the context of lignin valorization or the utilization of aromatic waste products.

del Cerro, C., et al. 2021. "Intracellular Pathways for Lignin Catabolism in White-Rot Fungi," *Proceedings of the National Academy of Sciences* **118**, e2017381118.

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Office of Science Initiatives

Real-Time Sensing and Adaptive Computing to Elucidate Microenvironment-Induced Cell Heterogeneities and Accelerate Scalable Bioprocesses

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Project Goals: The overarching goal of this project is to predict microbial performance in large-scale bioreactors by understanding cell population performance, metabolism, and cell-to-cell heterogeneity in simulated bioreactor microenvironments. Addressing the uncertainty gap in scaling between laboratory- and industrial-scale cultivations is key to accelerate innovation in the bioeconomy. This project will do so by developing and integrating computational and experimental tools, as well acquiring fundamental knowledge in microbial systems.

The biological conversion of renewable and waste sources to fuels and chemicals is an integral component of a sustainable bioeconomy. While biomanufacturing has been successfully demonstrated at the laboratory scale for a wide range of products, only a few have successfully been produced at industrial scales. This transition between laboratory- and industrial-scale cultivations represents a "valley of death" in biological processes, where uncertainties arise regarding the lack of predictability for microbial performance across scales. Mixing becomes one of the significant challenges encountered in large-scale bioreactors. In contrast to the well-mixed cultivations at the laboratory scale, large-scale cultivations are not uniformly mixed, resulting in uneven distribution of nutrients, pH, gas composition, and temperature. These heterogeneities impact microbial performance in an unpredictable manner, decreasing bioconversion efficiency and ultimately increasing manufacturing costs. Unless the capability to predict microbial performance in large-scale bioreactors can be realized, many biological conversion pathways will not come to fruition in the bioeconomy.

This multidisciplinary and multi-institutional project will develop and integrate experimental and computational tools and will acquire fundamental knowledge in microbial systems to address the uncertainty gap in scaling between laboratory- and industrial-scale cultivations. The group will establish a framework to predict and address the adequacy of a microbe at the beginning of the innovation cycle to mitigate risks during the development and scale-up of new bioprocesses. The team combines the strengths of metabolic engineering, fermentation science, systems biology, genome-scale modeling, automated high-throughput DNA sequencing, computational fluid dynamics, and machine learning. The knowledge gained through this work will serve as a foundation to address conversion issues at the microbial level and will extend to other biological disciplines that seek predictive understanding of multicellular system behavior (e.g., at an ecosystem level), which are relevant goals to BER.

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Field Observation of Water, Sediment, and Nutrient Distribution Patterns in Alluvial Ridge Basins Between the Abandoned Rio Grande Channels (Resacas)

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Project Goals: This project aims to understand mass accumulation processes in alluvial ridge (AR) basins in river deltas across scales by combining field observed and remotely sensed flow data from the Rio Grande Delta (RGD), Texas, with two numerical models, ANUGA (hydrodynamic) and dorado (particle transport). AR basins are topographic depressions bounded by abandoned deltaic channels that are natural depo centers for water, sediment, and nutrients. However, more is needed to know about such processes, as these areas comprise most of the broader populated deltas. Yet, previous studies have focused on understanding the infilling of channelized portions of a delta. Such a project on the RGD is much needed to not only fill in the knowledge gap for basic research but also provide the underserved community with such data to better understand how delta inundation patterns change to inform mitigation policies and engineering practices.

River deltas are net depositional landscapes that form at the coast, hosting populated socioeconomic hubs and providing essential ecosystem services, yet are facing stresses like land loss due to ongoing climate change and anthropogenic impacts. Channel avulsions, abrupt shifts in river course, are a main process in which the delta distributes sediment to build land in coastal regions. Previous works have focused on assessing water, sediment, and nutrient transport and deposition in channelized portions of the delta. Through multiple avulsions, however, deltas contain several generations of abandoned channels with high relict levees that bound topographic degressions, known as AR basins, accounting for most of the broader delta region. How mass accumulates in AR basins between the relict deltaic channels still needs to be understood. More importantly, climate change and natural disasters are shown to disproportionately affect underserved communities, especially those of the RGD in south Texas. Hosting ~1 million people, the RGD region has the lowest median household incomes and the highest health risks in the United States. The RGD region contains seven main abandoned channels of the Rio Grande, known locally as resacas. Despite frequent inundation due to extreme rainfall and storm surges, the water and sediment transport patterns of these AR basins remain elusive.

To fill this knowledge gap, researchers plan to deploy pressure sensors to measure water, nutrients, and sediment transport rates in three alluvial basins (Bahia Grande, Laguna Larga, and Vadia Ancha) spanning a range of potential sediment sources and tidal and human intervention conditions. Researchers will also collect sediment core samples throughout the alluvial basins and conduct radiocarbon age dating analysis to determine sediment and nutrient accumulation rates over multiple millennia. Researchers hypothesize that the accumulation rate is highest near the tidal inlet channels and decreases nonlinearly towards the basin interior. In addition, given the shallow flow depth, modern transport conditions in the AR basin are strongly correlated with spring and neap cycles, storm surges, and extreme wind events. The results of this study will be the first systematic field measurements of mass accumulation rates and patterns in AR basins of an extensive delta system, the RGD (i.e., the second largest river delta in the United States). Researchers also plan to use these data to develop a mechanical explanation of how river deltas fill AR basins and provide the underserved community with such data to better understand how delta inundation patterns change to inform mitigation policies and engineering practices.

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Understanding the Role of Duckweed Transcription Factor in Triacylglycerol Metabolism and Abiotic Stress Tolerance in Plants

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In nature, plant oils represent one of the most energy-rich sources of renewable hydrocarbons. They are stored as triacylglycerols (TAGs) or oils, which can be used as alternative feedstocks for biodiesel production. As an alternative feedstock, plant-based oils have several advantages over other fuels, including high energy content, no need for fermentation, compatibility with existing fuel technologies, and being environmentally friendly. Lifecycle analyses have shown that the production and use of jet fuel from oilseeds can results in lower greenhouse gas emissions as compared to petroleum-derived fuels. However, supplies of these

energy-rich oilseed compounds are limited due to low crop yield and limited available arable land. This project aims to use a combination of genomic, molecular biological, and biochemical analyses to explore transcription factor regulatory networks that regulate TAG/oil production in plants, and how they can be manipulated to increase carbon conversion to oils in oilseed plants.

Specifically, transcriptional regulators that have been found to increase oil content when overexpressed in *Arabidopsis* will be targeted to better understand the mechanism by which seed oil storage is enhanced. The central hypothesis of this project is that a transcription factor described in duckweed is also required to regulate TAG metabolism in Brassicaceae oilseed crops such as *Camelina*, canola, and pennycress. Three objectives will be pursued: (1) to determine requisite duckweed transcription factor functions in oil storage metabolism; (2) to alter oil content and agronomic performance in *Camelina*; and (3) to define an overall metabolic engineering strategy to leverage duckweed transcription factors for increasing TAG yield. Knowledge gained from this research will unlock new and creative avenues to use genetic engineering to enhance TAG in oilseed crops, which will help fulfill the world's growing fuel needs.

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Developing Temperature-Jump X-Ray Crystallography to Study Dynamic Biosynthetic Enzymes at Synchrotrons and X-Ray Free-Electron Lasers

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Project Goals: The overall goal of this project is to develop robust and accessible tools for measuring macromolecular dynamics at DOE X-ray lightsources. As a proof of concept, researchers are studying the catalytic mechanism of lipoxygenase enzymes, which operate via rate-limiting hydrogen tunneling.

Conformational dynamics underlie many important biochemical functions, such as enzyme catalysis, but they remain difficult to study. Rising to this challenge, the current generation of X- ray lightsources, including DOE synchrotrons and X-ray free-electron lasers (XFELs), offers new opportunities to study molecular motion using time-resolved crystallography. These instruments produce ultrafast, high-brilliance X-ray pulses that enable the observation of protein dynamics in real time following a rapid perturbation that synchronizes motion within the ensemble of crystallized molecules. To help make time-resolved crystallography a general tool that can be applied to study the dynamics of any protein of interest, the group is developing the use of infrared laser-induced temperature-jump (T-jump) as a rapid perturbation. This team has collaborated to pilot the use of new hardware and software for T-jump X-ray crystallography across structural biology facilities at SLAC National Laboratory. Development of T-jump crystallography methods synergize with the project's mechanistic studies of lipoxygenase enzymes, which catalyze carbon hydrogen (C-H) bond activation reactions. The rate-limiting step for lipoxygenase catalysis is the abstraction of hydrogen via a tunneling mechanism, which is hypothesized to be linked to conformational dynamics that modulate the donor–acceptor distance. Researchers are using T-jump crystallography to test this hypothesis by mapping the conformational dynamics of several lipoxygenase variants with different catalytic properties. This comparative analysis will lend insight into which of the observed motions are functional in catalysis and which are not. Experiments performed at both synchrotron Stanford Synchrotron Radiation Lightsource and XFEL Linac Coherent Light Source facilities will allow scientists to access broad timescales ranging from nanoseconds to milliseconds. Lipoxygenases are model systems for enzymatic C-H bond activation and prototypes for studying the role of tunneling in enzymatic reactions. This research will help shed light on the mechanistic details of how conformational dynamics promote function in these enzymes.

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Strengthening Educational and Research Capacity for Bioenergy Science at Alabama A&M University through a Combination of Education, Research, and Partnerships

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Project Goals: Alabama A&M University (AAMU) has an excellent record in the training of underrepresented individuals in the various disciplines of STEM. However, the university lacks the resources to initiate new programs or enhance current ones to address topics of national needs in some rapidly developing disciplines, including renewable energy. Therefore, through a combination of education, research, and partnerships with the DOE Joint Genome Institute (JGI) and Center for Advanced Bioenergy and Bioproducts Innovation (CABBI), the assembled team will execute a set of objectives to (1) build foundational capacity for bioenergy research by establishing and maintaining field trials of bioenergy crops as well as the genomics and phenomics tools to study them, (2) train underrepresented students at AAMU and provide networking opportunities to promote recruitment into the bioenergy workforce and/or graduate training programs, (3) establish and sustain interactions with JGI and CABBI partners [i.e., HudsonAlpha Institute for Biotechnology (HA) and University of Illinois Urbana Champaign (UIUC)] for consistent training opportunities, and (4) foster an inclusive and equitable role for underrepresented student trainees through mentorship to create a well-trained workforce as the nation transitions to a bioenergy economy.

In the initial phase of this educational project, efforts have been made in several areas:

- Student recruitment and engagement: Students have been recruited, both from within the university and from external institutions, to participate in various areas of bioenergy science. Two underrepresented undergraduate students (female) were recruited for microbiomics in partnership with JGI. Two underrepresented graduate students were recruited for plant genomics and biotechnology in partnership with CABBI and HA. Two trainees were recruited for high-throughput phenotyping/phenomics in partnership with CABBI and UIUC. These students are starting to engage with real-world projects and are being prepared for summer internships in 2024 at partner institutions.
- Partnership development and expansion: Initial efforts have been made to establish and expand interactions with JGI and CABBI partners. Specifically, regular meetings with the JGI team have been established. A formal joint training program between HA and AAMU is being developed and plans are underway for student engagement with UIUC.
- Training module development: Based on identified needs and gaps in student knowledge and skills, training modules are being designed. Modules cover literature review, data collection, and basic data exploration, with more to be developed for progressive learning.

The team will continue efforts in recruiting students for this project and other opportunities, enhancing their educational experience and preparing them for future careers in bioenergy science.

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BER-RENEW iSAVe: New Energy Sciences Workforce to Advance Innovations in Sustainable Arid Vegetation

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Project Goals: (1) Uncovering the natural principles that control greenhouse gas–capturing capacities of native arid soils; (2) defining key players of the C_1B community and evaluating their impact on soil–atmosphere exchange fluxes, soil chemistry, and water retention; (3) validating the applicability of C_1B supplements to benefit crop growth in nutrient-limited arid environments; and (4) evaluating impacts and constraints of the C_1B-based

technology implementation at midscale farm levels for energy crop production.

Sustainable use of arid soil will critically depend on the ability to foster natural principles that preserve soils and support the structure and functions of native ecosystem microbiomes in the long term. Native arid ecosystems represent an untapped reservoir of microbial functions essential for promoting plant growth and survival under water-limited and nutrient-poor conditions. The main goal of this study was to interrogate metagenomic data and functional capabilities of natural arid land microbiomes to design critical solutions for threatened agricultural land.

Here, the team presents a holistic study that started with thorough investigation of *in situ* methane (CH_4) fluxes and microbial communities inhabiting soil in the Anza Borrego Desert, a model arid ecosystem. Researchers found that *in situ* CH_4 fluxes indicate differences in the consumption of CH_4 between vegetated and unvegetated soil patches, reaching their peak on vegetated sites at the highest daylight times around noon, with up to 12 micromoles per square meter per hour. The metagenomic and enrichment studies revealed a ubiquitous presence of methanotrophic bacteria in the Anza Borrego Desert soil. The network analysis highlights the co-occurrence of CH_4-consuming bacteria (*Methylocaldum, Methylobacter,* and *Methylomicrobium*) and several members of Rhizobia and nitrifying bacteria. Sixty-one metagenome assembled genomes (MAGs) were generated, and eight MAGs were identified as methanotrophs, including four *Methylocaldum* spp. and *Methylobacter luteus*. Additional high-resolution sampling efforts revealed the co-occurrence of several methanotroph genera, of which the highest proportion corresponded to *Methylocaldum*, in both vegetated and unvegetated patches.

Several methanotrophic bacteria, most belonging to the genus *Methylocaldum,* were isolated and sequenced. Comparative genomic studies were carried out. The examination of the genome inventory of these strains found significant redundancy in primary metabolic pathways, including numerous copies of a key gene for methane oxidation and several genes for methanol oxidation, in addition to three pathways for one-carbon assimilation, and two strategies of carbon storage (glycogen and polyhydroxyalkanoates).

Furthermore, the interaction of native methanotrophic species with the California-native plant *Boechera depauperata* (Brassicaceae) were examined. When supplemented with methanotrophic traits, *B. depauperata* displays drought tolerance and increased growth as well as quantitative measures of plant resilience, including photosystem activity and increased leaf area. Metabolomic and transcriptomic analysis revealed promoting flavonoids in the plants and a decrease in various amino acids including tryptophan, followed by an upregulation in genes involved in the tryptophan-mediated indole-3-acetic acid biosynthesis pathway.

These results suggest a mutualistic relationship between *B. depauperata* and *Methylocaldum* sp., leading to higher plant drought tolerance. Data suggest that in this arid ecosystem, methanotrophs are associated with vegetation and the association might enhance native plant drought tolerance. This work provides essential evidence that the association between plants and methanotrophs in (semi)arid ecosystems plays a major role in supporting the vegetation diversity of those ecosystems that subsequently might be key for methane cycling, having a significant impact on the global levels of this potent greenhouse gas and ultimately influencing the global climate.

Funding Statement: Metagenomic and *Methylocaldum* spp. genome sequencing and assembly were carried out by the DOE Joint Genome Institute. This work was funded by the DOE Office of Science, Reaching a New Energy Sciences Workforce (RENEW) contract DE-SC0024289.

Designing Novel Enzymes for Complete Degradation of Recalcitrant Polyamides

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Project Goals: The project objectives are to (1) design enzymes capable of complete depolymerization of nylon 6 and nylon 66 and (2) engineer bacterial strains able to metabolize the degradation products to higher-value sustainable materials.

As of 2015, a total of 6.3 billion tons of plastic waste had been generated globally. It is estimated that only 9% of this total has been recycled, while 12% has been incinerated to recover energy values and the remainder has entered landfills. New technologies are needed to address this ever-growing problem. An alternative approach, harnessing the power of biology to not just depolymerize plastics back to their monomer precursors but convert them into higher-value products, offers stronger economic incentives and in turn would be expected to drive more rapid and widespread adoption. Toward that end, this group's work focuses on combining cutting-edge computational protein design and synthetic biology to address the challenge of complete biodegradation and upcycling of the recalcitrant polymers nylon 6 and nylon 66. Although natural enzymes have been shown to be able to degrade amorphous portions of polyamides such as nylon 6 and nylon 66, complete enzymatic degradation has not been demonstrated. Researchers hypothesize this to be due in large part to a lack of natural enzymes able to efficiently catalyze degradation of the crystalline portion of the polymer. To alleviate this limitation, the team is using a combination of physics-based and generative deep learning–based protein design methods to engineer new-and-improved enzymes with optimized active sites for binding and hydrolysis of polyamides. In conjunction, researchers are screening and engineering bacterial strains able to metabolize nylon 6 and nylon 66 degradation byproducts directly into central metabolism. Such platform strains can be used to produce a wide variety of fermentation products from central metabolites. Integration of nylon 6 and nylon 66 depolymerizing enzymes into these engineered hosts will provide a novel, elegant, and cost-effective consolidated fermentation process for nylon upcycling to higher-value sustainable materials.

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BIOIMAGING

University Project

Probing the Mechanisms of Microbial-Mediated Polymer Deconstruction on the Molecular- and Systems-Level

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Project Goals: The microbiology-based projects within the UCLA-DOE Institute employ molecular, biochemical, genome sequencing, and *in silico* approaches to better understand biological processes that drive carbon recycling in nature. These findings impact multiple areas of BER interest, including bioconversion of model substrates in natural and manmade environments, the associated biochemistry of key degradative enzymes, and the design of plant-based biomass deconstruction strategies for biobased chemicals production. Genomics programs that instruct metabolic pathways for key substrates are being elucidated in both model and novel microbe systems. Next-generation omics methods are being applied to interrogate environmentally relevant pathways, as well as their interactions with defined microbial communities. In related work, the project seeks to define the pathways used by cellulolytic microbes to degrade lignocellulose and other polymers. Using a combination of experimental and bioinformatics approaches, researchers seek to learn how anaerobic microbes sense environmental changes that induce the synthesis and assembly of extracellular cellulosome-like structures that degrade different types of plant biomass. Collectively, the results of these basic science studies provide fundamental insight into processes that drive carbon recycling and will facilitate the development of new microbial-based methods to produce renewable chemicals and materials from abundant biomass.

Elucidation of microbial pathways for metabolism and degradation of model polymeric substrates. Genomic, proteomic, and informatic studies were performed on model and newly identified microbes to elucidate how representative plant-derived substrates are efficiently metabolized. Here, core pathway enzymes for polymers, sugars, and fatty acids are being investigated and further characterized in a spectrum of cellulolytic microbial species. Recombinant, structural, and informatic studies of key enzymes in these pathways were performed to explore thermodynamic rate-limiting steps during anaerobic cell growth. Associated electron transfer pathways needed for hydrogen and formate production by polymer degrading microbes were also examined.

In complementary studies, proteomic and mass spectrometry methods were performed to further characterize metabolic pathways, protein post-translational modifications (PTMs), and cellular envelope components of model polymer-degrading microbes. Characterizing enzyme-disrupting PTMs will help decipher their relationship with the metabolism of biomass by microbial strains. The team has discovered that acyl-lysine modifications arising from reactive metabolites are strikingly abundant in model beta-oxidation bacteria. Acetyl, butyryl, 3-hydroxybutyryl, and crotonyl modifications were observed in a range of species including *Syntrophomonas wolfei* and *Syntrophus aciditrophicus*. Interestingly, the types of modifications that occur are correlated with the complexity of the carbon substrate, and the relative abundance of these modifications significantly change in response to different carbon sources. For example, *S. wolfei* subsp. *methylbutyratica* is capable of metabolizing longer carbon substrates displaying diverse methylbutyrylation, valerylation, and hexanoylation modifications.

Probing how bacteria produce cellulosome-like structures for efficient plant polymer degradation. The project's efforts are focused on elucidating how these bacteria sense different types of biomass to optimize the enzyme composition of the cellulosome and gaining broad insight into how cellulosomes are assembled through comparative genome analyses. Here, the team reports recent results that suggest biomass-sensing membrane receptors undergo autoproteolysis via a succinimide intermediate, thereby predisposing them to biomass-induced dissolution triggering gene expression changes in cellulosomal genes. Ongoing efforts are focused on using a transcriptomics-based approach to map the full set of genes controlled by each receptor and to determine the biomass signals which they detect. Finally, the team presents the initial results of a comprehensive analysis of published sequenced genomes from which novel cellulosome-displaying bacteria have been identified. The results of this work will shed light onto the diversity of cellulosome architectures present in biology and could facilitate engineering efforts for designer recombinant cellulolytic bacteria.

* Presenting Author

To explore pathways for plant-derived polymer deconstruction, PacBio long-read sequencing approaches were used to sequence, assemble, and annotate genomes of previously isolated bacterial strains that utilize such substrates when grown in pure cultures or cocultured with suitable microbial partners. The project is also extending gene annotation methods beyond the standard homology-based interferences of these microbial genomes based on co-evolution, such as phylogenetic profiling. To this end, researchers are generating maps of domain interactions by leveraging the data found in UniProt, allowing the mapping of interactions between thousands of domains based on their conservation across 10,000 prokaryotic genomes. The team is developing web-based tools to visualize these interactions and navigate relationships between bacteria.

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National Laboratory Projects

Development of High-Throughput Methods to Assist Measure of Biological Nitrification Inhibition in *Populus* Soils

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Project Goals: This project seeks to discover and validate gene functions and pathways in *Populus trichocarpa* that lead to generation of biological nitrification inhibitors and to understand their roles affecting nitrogen use efficiency (NUE).

Plants have evolved to mediate nitrification processes by exuding biological nitrification inhibitors (BNIs) in soil to alter the functions of the plant-associated microbiome. BNIs have emerged as one route to mitigate loss of nitrogen (N) and improve NUE. Little is known of the genes, gene families, and associated pathways related to BNI production and function. Thus, experimental data that comprehensively link host traits, genetics, and exudation of BNI compounds are needed. Such data are critical to creating systems network models that can deconvolute complex host–microbiome interactions, system feedbacks on the microbiome, and impacts on biogeochemical cycles. This project seeks to discover and validate gene functions and pathways in *P. trichocarpa* that lead to generation of BNIs and understand their roles affecting NUE. To begin, methods and protocols are needed to measure nitrification traits in soil obtained from a *P. trichocarpa* genome-wide association study population. To accurately characterize such processes, measurements of gross nitrification rate (GNR) using ^{15}N-isotope dilution techniques are needed. Unfortunately, current assays can be costly and difficult to implement and are relatively low-throughput. Linking plant genomic function to nitrification inhibition activity requires measuring GNR phenotypes across a diverse population comprising hundreds and thousands of samples, hence the need for alternate methods. Here, chemical derivatization protocols are adapted to enable isotope dilution measurements with high throughput and sensitivity.

Methods were developed to capitalize on high-throughput capabilities of an immediate drop-on-demand (I.DOT) system coupled with open port sampling interface–mass spectrometry (OPSI-MS). Derivatization products were

analyzed by dispensing 10 nanoliters of diluted (water) samples into a flow of solvent, which is subsequently characterized by mass spectrometry. Reactions were optimized for reaction time, derivative concentration, and other variables. Soil samples were kept refrigerated until supplemented with ^{15}N-rich ammonium sulfate or potassium nitrate, incubated for 24 hours, and extracted with 1 molar potassium chloride. Measure of nitrate was achieved by modifying derivatization protocols of 2,3-diaminonaphthalene. Using the I.DOT/OPSI-MS system, the reaction could be monitored over time using various 2,3-diaminonaphthalene concentrations. Without a concentration step, a limit of detection (LOD) of low micrometer was achieved for ^{14}N-2,3-naphthotriazole (NAT) and even lower for ^{15}N-NAT. O-phthaldialdehyde (OPA), dansyl chloride (DAN), and 9-fluorenylmethoxycarbonyl chloride derivatization reactions were explored for detection of ammonia. Of these, OPA resulted in the highest sensitivity with the simplest derivatization procedure. An LOD of low micromolarity was achieved using OPA. Both DAN and OPA methods had <10% coefficient of variation. DAN and OPA methods were used to demonstrate measures of GNR and gross mineralization rate in soil samples collected near *P. trichocarpa* with varying concentrations of N (milligrams ^{15}N per kilogram of soil). Notably, the DAN and OPA methods were able to quantitively relate ^{15}N/^{14}N isotope ratios using high-resolution mass spectrometry or selected multiple reaction monitoring with 3 seconds per sample throughput and no sample cleanup. Together, these methods represent a simple, flexible, and fast approach for measuring nitrate and ammonia in soil extracts.

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Profiling Temporal and Spatial Activities of Rhizobacteria

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Rhizobacteria play significant roles in influencing plants through symbiosis and virulence. However, there is limited understanding of rhizobacterial activities in the face of temporary and spatially changing environments within the rhizosphere. In this study, researchers aim to develop novel experimental approaches to characterize temporal and spatial activities of root-colonizing *Pseudomonas* strains. In the first project, the team developed a massively parallel reporter assay, which employs DNA barcode as a reporter of promoter activity, in *P. simiae* WCS417. This approach allowed researchers to characterize *in planta* promoter activities of *P. simiae* without the need to remove an overwhelming amount of plant RNA. In the second project, researchers are working on generating an engineered *P. putida* KT2440 that harbors a chemoreceptor library. Using the developed strain, researchers aim to screen chemoreceptors functionally important for plant root colonization. These engineering strategies will be valuable to investigate plant–microbial interactions and may provide new insights to manipulate microbial systems and enhance plant productivity.

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BIOPREPAREDNESS

Unveiling Molecular Interactions and Metabolic Contributions in Sorghum Anthracnose Defense: Towards the Integration of Fungal Pathogen and Host Sorghum Models

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Project Goals: To gain deeper insights into the virulence mechanisms of the fungus *Colletotrichum sublineola* in anthracnose, it is essential to comprehend the pivotal metabolic interactions occurring between *C. sublineola* and its host organism, sorghum. Through the development of mechanistic models, researchers can delve into these crucial metabolic interactions, shedding light on the roles of both the fungus and the host sorghum in the process.

Anthracnose, a devastating disease in sorghum, is caused by the hemibiotrophic fungal pathogen *C. sublineola* (Crouch and Tomaso-Peterson 2012). Sorghum's nucleotide-binding leucine-rich repeat proteins (NLRs) play a pivotal role in recognizing virulence effectors of pathogens like *C. sublineola*, triggering effective immune responses vital for plant defense (Rausher 2001). Nonetheless, the intricate molecular interactions governing *C. sublineola* pathogenicity, anthracnose resistance, and susceptibility in sorghum remain poorly elucidated. Further, *C. sublineola* metabolism during its appressoria and haustoria invasion stages is not well understood. To address these knowledge gaps, the overall goal within the wider context of the BRaVE project is to build a metabolic model of the interactions between sorghum and *C. sublineola*. To this end, the team sequenced, assembled, and annotated a newly isolated *C. sublineola* pathogen, followed by the reconstruction of a genome-scale metabolic model (GEM) around a well-curated core of central carbon metabolism, fermentation, electron transport chains, and energy biosynthesis. A comparative study was also performed between the new *C. sublineola* genome and many existing nearby fungal strains.

This GEM reconstruction builds upon extensive curation efforts to develop an improved fungal modeling template for the DOE Systems Biology Knowledgebase (KBase) by consolidating and reconciling data from 13 diverse published fungal models (Edirisinghe et al 2023). This integration involved harmonizing the biochemistry of each published model with the ModelSEED biochemistry database, effectively minimizing redundancy and inconsistency in biochemical pathways and the underlying protein annotations.

This approach is further supported by protein family data computed across the 13 fungal species and a set of additional well-sequenced fungal strains mapped to pertinent biochemistry data. This system systematically captures the unique biochemistry of each model, ensuring consistency in annotation mapping with the relevant biochemical context.

The curated and reconciled pathways, refined and consistently annotated protein families, and enhanced fungal template model are bundled together within the readily accessible user-friendly Build Fungal Model application on the KBase platform (https://narrative.kbase.us). The work also resulted in significant improvements to all the published fungal models that were reconciled in the efforts to build the fungal modeling tool. Additionally, researchers present a KBase narrative workflow illustrating construction of the central carbon model for *C. sublineola* utilizing data from its newly sequenced genome and other closely related genomes.

For the plant side of the eventual host–pathogen mechanistic model, researchers applied the PlantSEED (Seaver et al. 2018) approach to generate a high-quality metabolic model of sorghum using the latest set of gene models. Previously, the conservative approach of predicting orthologs using OrthoFinder meant researchers had a high rate of false negatives. Researchers adopted a new approach that takes into account the distribution of pairwise sequence identity for each protein family, allowing both orthologs and in-paralogs to be predicted as carrying the functions of plant primary metabolism, enabling researchers to assign sorghum enzymes to an additional 10% of plant primary metabolism.

Researchers are actively applying the fungal and plant models independently to integrate and mechanistically interpret multiomics datasets produced by other project team members, while the ultimate goal is to merge these models into a predictive dynamic host–pathogen interaction model.

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Low-Dose Radiation Research

Multiscale Computational Digital Twins for Whole-Body to Subcellular Radiation Effects

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Project Goals: The goal of the work is to advance understanding of the contributions of low-dose (LD) radiation to cancer by identifying and modeling the key molecular and cellular mechanisms involved, developing optimal strategies for interrogating these mechanisms experimentally, acquiring and integrating diverse datasets to formulate and test hypotheses, and validating predictive multiscale models of radiation risk. The work will be performed through three broad goals: (1) identifying and modeling molecular and cellular mechanisms of LD radiation damage and repair; (2) identifying and characterizing signatures of LD exposure and LD-induced tumorigenesis; and (3) developing a multiscale modeling and simulation framework for estimating cancer risk from radiation exposures.

This work describes the development and implementation of a multiscale computational digital twin framework to assess radiation exposure effects on humans. Using high-performance computing methods, the team assess radiation exposure effects at the whole-body, multicellular, and subcellular scales, seamlessly integrating among the three. The project's framework includes a population of human digital phantoms along with computing environments to model radiation transport and associated radiation damage. The project includes a multiscale perfusion model for radioisotope delivery and uptake, modules for DNA damage and DNA repair, and multicellular growth models to determine radiation effects on cells and organs. An explanation of the integration of these scales into the framework is provided below.

At the subcellular level, using experimental Hi-C data (Sanders et al. 2020), researchers create 3D chromosome models imported into TOPAS-nbio (a Monte Carlo simulation platform) to estimate DNA double-strand breaks (Chatzipapas et al. 2020). Mechanistic repair models from the Mechanistic DNA Repair and Survival Model (MEDRAS) predict post-radiation aberrations over time (McMahon and Prise 2021). Validation against *in vitro* studies and cell experiments with a mouse breast cancer cell line confirms accuracy.

At the multicellular and whole-body scales, the project integrates a tumor growth model in Compucell3D with eXtended CArdiac Torso (XCAT) phantoms for whole-body simulations (Segars et al. 2013). Geant4, a Monte Carlo simulation software, calculates absorbed dose in radiation therapy protocols, feeding back into Compucell3D to assess cell survivability (Allison et al. 2006, 2016; Swat et al. 2012). Validation against recent multicellular models shows promising results, with ongoing efforts to validate against spheroid-based experiments.

For multiscale perfusion modeling, the team has implemented a physiology-based pharmacokinetic model

simulating radioisotope distribution at the organ level, feeding into a computational fluid dynamics model for tissue-level perfusion. Work is underway to demonstrate subcellular and multicellular scale radiopharmaceutical perfusion. Preliminary results involve growing spherical tumors with multiple cell types in a controlled nutrient environment, exposing them to Actinium 225 in Geant4 to estimate cell radiation dose and survivability.

In conclusion, the project's framework offers a robust tool for modeling radiation effects across various radiation dose–exposure scenarios. Importantly, parts of this pipeline are fully automated and optimized for graphics processing unit computation. With potential applications in environmental radiation exposures, occupational exposures, and medical exposures including radiation treatments, the team envisions the multiscale *in silico* digital twin framework providing a robust method to evaluate outcomes from unwanted exposures, as well as the ability to optimize desired exposures (such as radiation treatments) for the benefit of the individual. This framework facilitates predicting cell survival and treatment outcomes across different cancer types, integrating absorbed dose, biodistribution, cell toxicity, and repair mechanisms to determine overall outcomes in the human body.

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Artificial Intelligence Foundation Models for Understanding Cellular Responses to Radiation Exposure

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Project Goals: Development of a machine-learning pipeline which identifies key morphological features in cells treated with low-dose radiation.

The use of machine-learning models in cellular biology has drastically increased with rapid advances in artificial intelligence. These models, trained on cellular images, often analyze thousands of different features of an image of cells, from the number of cells to nucleus diameter. However, more research is needed on the use of vision transformers for cellular classification models.

Vision transformers use techniques different from other image classification models, such as convolutional neural networks. Vision transformers, for example, do not run the source image through various data augmentation layers but instead segment the original image into multiple patches, which are then linearly passed through the transformer. Many vision transformers have been developed in recent years, demonstrating promising validation accuracy when classifying large numbers of images; for the purposes of this project, researchers have implemented the MURA vision transformer, an efficient version that has shown reliable validation accuracy.

While there are many applications for the use of vision transformers when analyzing cellular images, for the purposes of this study, researchers have focused on the analysis of human umbilical vein endothelial cells (HUVEC),

which have undergone low-dose radiation exposure. A large amount of research has been done on the effects of acute, high doses of radiation on the morphological profile of human cells. However, the effects of low-dose radiation on cellular morphology have yet to be studied. If phenotypic features of HUVEC cell morphology can be identified using a vision transformer, it can lead to advanced and more efficient screening for low-dose radiation exposure.

Image data from the JUMP-Cell Painting Consortium was used to develop and fine-tune the vision transformer pipeline. The JUMP-Cell Painting Consortium is a collaboration between multiple laboratories to create a large repository of publicly available cell painting images. More specifically, the consortium contains approximately 115 terabytes of images of human osteosarcoma (U2OS) cells that have undergone either a chemical treatment or genetic perturbation and then have had cell painting performed on them for imaging. The size, consistency, and variety of this dataset provide an excellent benchmark for the MURA model's validity and the developed pipeline's effectiveness.

To provide clean and accurate data for the vision transformer model, the group performed cell segmentation on the cell painting images before training the model. The process of cell segmentation involves identifying the borders of each individual cell within the source image and extracting it so that each cell is contained within its own image to be fed into the model. This ensures factors, such as the number of cells or clustering, are not considered when training the vision transformer. The CellProfiler application was integrated into the group's pipeline to perform cell segmentation. CellProfiler is a widely used software designed for biological image processing. For the purposes of this project, researchers developed a pipeline that will stack each image channel of the cell painting images; identify the cellular components; and segment and export the cell images into the group's vision transformer pipeline.

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Molecular and Cellular Responses of Human Endothelial Cells to Low-Dose Radiation

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Project Goals: The biological impact of low-dose radiation exposure remains an important open question in radiation biology research, with significant implications for human health risk assessment, policy, and regulations. This project is leveraging advances in AI, high-throughput experimental technologies, and multiscale modeling and simulation to advance scientific understanding of the molecular and cellular processes involved in low-dose radiation and cancer risk, accelerate discovery, and connect insights across scales.

Radiation exposure has a wide spectrum of impacts on human health, notably in carcinogenesis but also in neurological and cardiovascular disorders. While acute toxicity from high doses of radiation is well-characterized, understanding the range of outcomes following exposure to low-dose radiation is more challenging. This project is establishing new experimental workflows that will enable high-throughput experiments across molecular and cellular scales to facilitate more comprehensive modeling.

In a pilot study, a monolayer of human umbilical vessel endothelial cells (HUVECs) was exposed to a point source of ^{137}Cs at a low dose rate of 6 milligrays (mGy) per hour. Cells were exposed for one week in culture (i.e., 1,008 mGy total dose) and then harvested for RNA or replated for Cell Painting staining.

Cell Painting is a streamlined multi-parameter approach to fluorescence microscopy that provides rich feature data of cell structure and function. A major advantage of Cell Painting is a robust publicly available dataset spanning thousands of small molecular and genomic perturbations produced by the collaborative JUMP Consortium. The scale of characterized phenotypes has facilitated development of predictive models that incorporate chemical structural information, biological mechanism of action, and gene expression, which will be expanded into the realm of radiation exposure.

With Cell Painting, features can be extracted based on staining of the nuclear and endoplasmic reticulum plasma membranes and cellular Golgi, actin, nucleoli, and mitochondria. Principle component analysis of control and irradiated cells provided a proof-of-principle demonstration

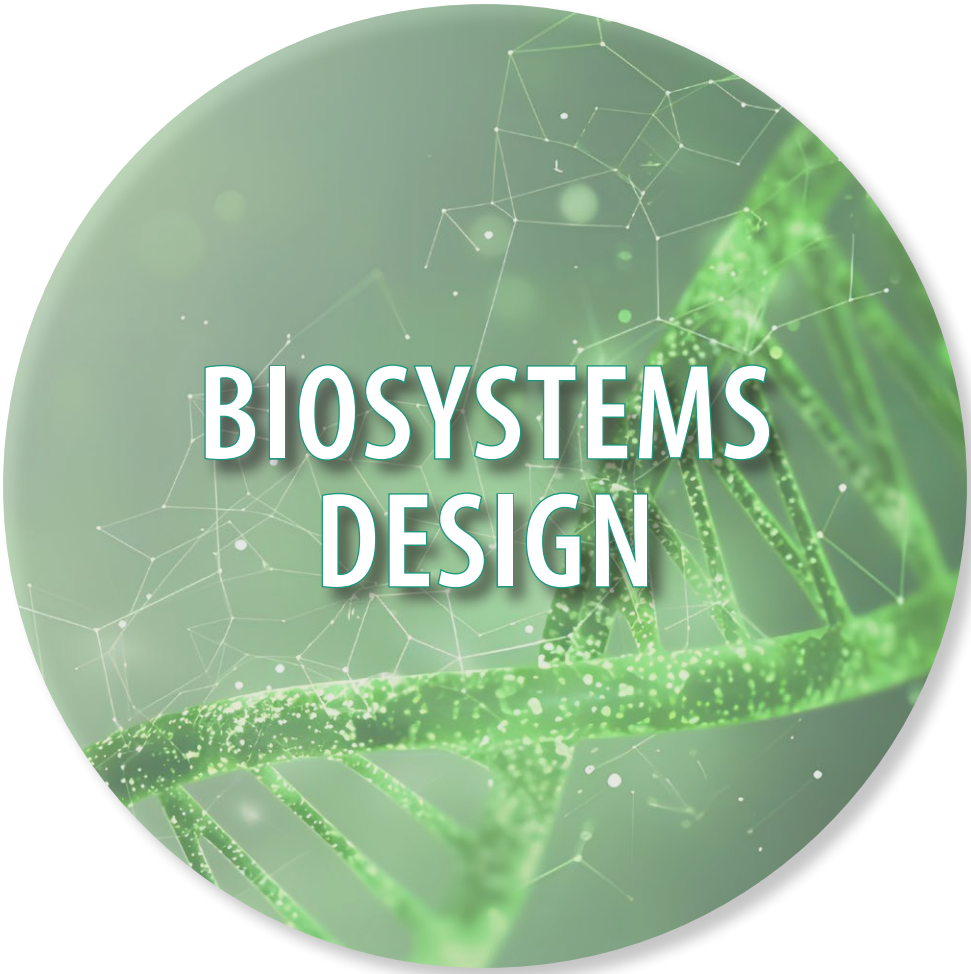
that Cell Painting enables detection of features impacted by irradiation. Transcriptome analysis revealed that in endothelial cells, radiation robustly induced cell response pathways integral to cytokine and chemokine pathways, such as the Tumor Necrosis Factor (TNF) pathway.

Underscoring the relevancy of HUVECs to cardiovascular disease, pathways associated with "lipid" and "atherosclerosis" were also activated. Two Kyoto Encyclopedia of Genes and Genomes terms shed light on the molecular mechanisms of these processes, namely the HIF-1 and NF-kappa B signaling pathways.

To compare these results to previous studies of low-dose radiation exposure, data were compared with gene expression datasets from the RadBioBase, a publicly available comprehensive transcriptome repository of irradiated mammalian samples. Datasets that used human cells and doses below 0.5 Gy were selected to identify 235 genes impacted by radiation across four published datasets. Of these, 35 genes were also seen in the data, notably the inflammatory cytokines IL6 and IL1B, as well as the genes PTGS2 (COX2) and CXCL12, which are involved in inflammatory processes underlying cardiovascular disease.

To overcome the limitations (e.g., variable dose field and high activity) of the point radiation source in the pilot study, a major goal of the next project phase is to prototype and deploy new source geometries in a 96-well plate format for high-throughput experimental exposures. New source geometries will require minimal activity, provide uniform dose fields, and enable multiple dose-rate exposures in parallel. The impact of low-dose radiation will then be assessed with molecular (e.g., multiomic) and cellular (e.g., Cell Painting) assays to develop advanced multiscale models of low-dose radiation impacts.

Funding Statement: Argonne National Laboratory's work on the Low-dose Understanding, Cellular Insights, and Molecular Discoveries (LUCID) program was supported by the DOE Office of Science, BER program, under contract DE-AC02-06CH11357.


BIOSYSTEMS
DESIGN

University Projects

Engineering C_4 Feedstock Crops for Improved Water Use Efficiency and Drought Avoidance

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https://www.harnessh2optimizecrops.org/

Project Goals: Bioenergy feedstocks need to be deployed on marginal soils with minimal inputs to be economically viable and have a low environmental impact. Currently, crop water supply is a key limitation to production. The yields of C_4 bioenergy crops such as *Sorghum bicolor* have increased through breeding and improved agronomy. Still, the amount of biomass produced for a given amount of water use (water use efficiency, or WUE) remains unchanged. Therefore, the project aims to develop novel technologies and methodologies to redesign the bioenergy feedstock sorghum for optimal WUE. Within this broader context, this subproject is using *Setaria viridis* as a rapid-cycling model for gene discovery. Researchers aim to develop and demonstrate novel methods and resources to accelerate both the production of genetic variants and phenotyping of WUE traits as part of reverse and forward genetics approaches to discover genes regulating stomatal patterning and WUE.

To aid in biosystem design for improvement of WUE and drought avoidance, researchers have recently investigated (1) gene expression patterns during grass stomatal development, (2) impacts on internal leaf anatomy (i.e., intercellular air spaces) and gas exchange associated with variation in stomatal density, and (3) stacking greater leaf WUE with improved root systems.

Some orthologs of *Arabidopsis* genes implicated in stomatal development behave differently in sorghum, potentially a result of the co-option of genes into the construction of a more complex stomate or a result of the difference between the grass cell-file-system epidermis versus the *Arabidopsis* puzzle-piece epidermis. Candidate genes and promoters were identified for use in targeted expression of the transgene that drives lower stomatal density and greater WUE.

High-resolution micro-computed tomography revealed stark differences between the airspaces below stomata on the adaxial versus abaxial leaf surfaces, with major potential implications for fluxes of carbon dioxide (CO_2) and water. This may drive changes in mesophyll conductance that support greater WUE than if stomatal conductance alone was modified. Meanwhile, a survey of accessions varying in stomatal density from four sorghum races found that mesophyll conductance to CO_2 is independent of stomatal density and positively influences intrinsic WUE.

Root system size in bioenergy grasses is determined by initiation and growth of crown roots, a type of shoot-borne root. Researchers characterized a novel genetic locus, crown root defective (CRD), that is necessary for crown root growth under well-watered conditions. Molecular characterization reveals that *CRD* encodes a WD-40 repeat protein that acts as a scaffold and physically interacts with proteins controlling hormone signaling. Physiological characterization suggests that *CRD* promotes crown root growth by suppressing ethylene biosynthesis.

Funding Statement: This research was supported by the DOE Office of Science, BER program, grant numbers DE-SC0023160 and DE-SC0018277.

Sequencing-Driven Accelerated Discovery of Genes Regulating Water Use Efficiency and Stomatal Patterning in C_4 Crops with High-Throughput Phenotyping

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Project Goals: Bioenergy feedstocks need to be deployed on marginal soils with minimal inputs to be economically viable and have a low environmental impact. Currently, crop water supply is a key limitation to production. The yields of C_4 bioenergy crops such as *Sorghum bicolor* have increased through breeding and improved agronomy. Still, the amount of biomass produced for a given amount of water use (water use efficiency, or WUE) remains unchanged. Therefore, the project aims to develop novel technologies and methodologies to redesign the bioenergy feedstock sorghum for optimal WUE. Within this broader context, this subproject is using *Setaria viridis* as a rapid-cycling model for gene

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discovery. The goal is to devise novel methods and develop resources to create genetic variations and streamline the phenotyping of WUE traits. These advancements are crucial for their application in forward genetics approaches aimed at identifying genes that regulate stomatal patterning and WUE.

Stomata regulate the exchange of carbon dioxide (CO_2) and water vapor between the leaf and atmosphere, and therefore play a key role in determining WUE. Relatively little is known about the genes that regulate stomatal patterning and WUE in C_4 grasses. To advance efforts to engineer improved WUE of bioenergy crops, researchers are developing novel methods to accelerate the use of forward genetics for gene discovery. The team conducted a forward genetic screen of 340 families of a nitrosomethylurea-mutagenized *S. viridis* population, of which 185 lines were selected for having interesting visual phenotypes in a pre-screen. Researchers assessed whole-plant WUE using imaging and automated lysimeters and then collected leaf sections to screen for abnormalities in stomatal patterning. High-throughput optical tomography imaging was utilized to generate high-resolution images of the leaf surface. Researchers utilize a machine learning model for identifying the size, shape, and number of stomata in *S. viridis*. Seventy families of the first 155 families were identified to segregate for WUE and/or stomata mutant phenotypes and therefore selected for a second screen. Seeds harvested from 40 families were confirmed as fixed WUE and/or stomata mutants. These families are part of a larger population which are being sequenced by the DOE Joint Genome Institute (JGI) to create a sequence-indexed mutant population. DNA isolated from representatives of each family, or the mutant segregants from a family, is being sequenced by JGI to identify disruptive single-nucleotide polymorphisms (SNPs) in candidate genes. Preliminary analysis suggests that each line contains an average of 266 disruptive polymorphisms, with ~15 classified as high impact. The work demonstrates high-throughput phenotyping and genotyping strategies to quickly identify genes of interest, followed by verification of genes through a transgenic approach for a role in WUE. Success in this effort could be leveraged to accelerate research on a wide range of other traits and species.

Funding Statement: This research was supported by the DOE Office of Science, BER program, grant numbers DE-SC0023160 and DE-SC0018277.

Enabling Synthetic Biology in *Setaria* and *Sorghum* Through Targeted Mutagenesis and Programmed Transcriptional Regulation

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Project Goals: Bioenergy feedstocks need to be deployed on marginal soils with minimal inputs to be economically viable and have a low environmental impact. Currently, crop water supply is a key limitation to production. The yields of C_4 bioenergy crops such as *Sorghum bicolor* have increased through breeding and improved agronomy. Still, the amount of biomass produced for a given amount of water use (water-use efficiency, or WUE) remains unchanged. Therefore, this project aims to develop novel technologies and methodologies to redesign the bioenergy feedstock sorghum for optimal WUE. Within this broader context, this subproject is using *Setaria viridis* as a rapid-cycling model for gene discovery. Researchers aim to develop and demonstrate novel methods and resources to accelerate both the production of genetic variants and phenotyping of WUE traits as part of reverse and forward genetics approaches to discover genes regulating stomatal patterning and WUE.

Improving WUE in sorghum requires the ability to manipulate endogenous genes and gene expression patterns. Researchers are implementing several technologies for the genetic improvement of sorghum as well as the model C_4 plant *S. viridis*. In both plants, the delivery of DNA to cells is critical to alter the genetic code. Researchers currently have a robust pipeline for *S. viridis* transformation; however, traditional methods of sorghum transformation are laborious and time consuming. To expedite sorghum transformation, researchers are using developmental regulators, including BABY BOOM and WUSCHEL2, to promote somatic embryogenesis from transformed sorghum leaf cells. Using traditional transformation methods in *S. viridis*, researchers have succeeded in making specific nucleotide substitutions through prime editing. This technology enables precise small insertions (~30 base pairs) or deletions (~100 base pairs). Researchers are currently focused on introducing changes that would alter the kinetic activity of the key photosynthetic enzyme, phosphoenolpyruvate carboxylase.

To accelerate gene editing, researchers are using RNA viruses to deliver gene editing reagents through infection. Because the cargo capacity of most plant RNA viruses is limited to approximately 1 kilobase, researchers have made transgenic lines of sorghum and *S. viridis* that express Cas9; single-guide RNAs are expressed from the virus and gene editing occurs through infection. The current goal is to use viruses to edit the germline so that seeds can be harvested with heritable modifications to their genomes. To achieve precise control over gene expression, researchers are building synthetic genetic circuits to enable spatial and tissue-specific control over gene expression. Synthetic circuits offer a means to reprogram plant development and control growth. Finally, to expedite classical genetics, researchers have generated a male sterile line of *S. viridis* using CRISPR-Cas9 by making targeted, inactivating mutations in a gene important for pollen development. These male sterile lines are currently being tested and promise to greatly accelerate genetic analyses in *Setaria*.

Funding Statement: This research was supported by the DOE Office of Science, BER program, grant numbers DE-SC0023160 and DE-SC0018277.

Leveraging Leaf Structure and Biochemistry to Enhance Water Use Efficiency in Sorghum

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https://www.harnessh2optimizecrops.org

Project Goals: Bioenergy feedstocks need to be deployed on marginal soils with minimal inputs to be economically viable and have a low environmental impact. Currently, crop water supply is a key limitation to production. The yields of C_4 bioenergy crops such as *Sorghum bicolor* have increased through breeding and improved agronomy. Still, the amount of biomass produced for a given amount of water use (water use efficiency; WUE) remains unchanged. Therefore, this project aims to develop novel technologies and methodologies to redesign the bioenergy feedstock sorghum for optimal WUE. Within this broader context, this subproject is using *Setaria viridis* as a rapid-cycling model for gene discovery. The team's goal is to devise novel methods and develop resources to create genetic variations and streamline the phenotyping of WUE traits. These advancements are crucial for their application in forward genetics approaches aimed at identifying genes that regulate the efficiency of the carbon-concentrating mechanism (CCM) and WUE.

At the whole-plant scale, WUE is defined as biomass production per unit of water loss through transpiration. Plant WUE is largely determined by leaf-level "intrinsic" WUE, which is defined as the ratio of photosynthetic carbon gain (A_{net}) to stomatal conductance to water vapor (g_s). The intrinsic WUE in C_4 plants, such as sorghum, is generally high because they use a CCM to increase A_{net} while maintaining low g_s. However, under drought conditions, A_{net} can be limited by insufficient supply of carbon dioxide (CO_2) to drive the CCM. The focus of the research presented here is to enhance intrinsic WUE in sorghum by (1) increasing the conductance of CO_2 within the leaf to overcome reduced g_s and (2) to enhance the catalytic efficiency of the first committed reaction of the CCM catalyzed by phosphoenolpyruvate carboxylase (PEPC).

The conductance of CO_2 within the leaf's mesophyll is partially determined by cell wall structural polymers that influence wall thickness and porosity. The group has demonstrated that changes in cell-wall mixed-linkage glucans and ferulic/coumaric acids influence leaf CO_2 and water conductance, which led to an increase in whole plant WUE. This research team is also using a forward genetic screen to identify other key genes that influence traits related to the internal conductance of CO_2 and leaf intrinsic WUE.

Additionally, researchers have determined that variation in the affinity of PEPC for bicarbonate (HCO_3; K_{HCO3}) across several C_4 species is sufficient to increase modelled A_{net} under low g_s that occurs during drought. Researchers have further demonstrated, using a heterologous *Escherichia coli* expression system, that they can engineer enhanced *in vitro* PEPC kinetic properties with specific modifications to key amino acid residues. These modifications are also predicted through models of C_4 photosynthesis to increase photosynthesis under low g_s and enhance WUE. To translate these findings into plant systems, the team is using prime editors to create heritable edits of these key amino acid residues. Researchers are currently phenotyping plants engineered with these modified PEPCs to determine the impact on intrinsic and whole-plant WUE.

Funding Statement: This research was supported by the DOE Office of Science, BER program, grant numbers DE-SC0023160 and DE-SC0018277.

Developing Chassis for Low Density Polyethylene Upcycling from Microbes Native to the Gut Microbiome of Yellow Mealworms

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Project Goals: This project aims to enable the efficient deconstruction of polyolefins and upcycling to itaconic acid. The team will use novel genomic insights into nutrient-enhanced polyolefin degradation by the yellow mealworm gut microbiome and genetic tool development for gut microbiome isolates and engineered microbial communities.

Annually, more than 200 million tons of plastic waste in the form of polypropene, high density polyethylene, and low density polyethylene (LDPE) are generated and accumulate in the environment. No robust system exists to capture this carbon; however, in prior work, researchers identified myriad upregulated nonmodel species in plastic-fed mealworm guts. Moreover, team members previously showed that gut isolates from these genera grow on LDPE as their primary carbon source and chemically modify LDPE films upon inoculation. These taxa have been identified as upcycling chassis for development due to their prevalence in plastic-enriched microbial communities.

As a prerequisite for targeted genetic engineering, the research team collected genome and methylome sequence data of eight gut isolates that can grow on LDPE as their primary carbon source. The research group successfully identified methylation motifs in each of the eight isolates and have located and annotated methyltransferases in the genome of each strain. *Escherichia coli* strains capable of producing plasmids with these tailored methylation patterns greatly facilitate transformation and genetic engineering for future community engineering efforts.

To further understand the role of microbial isolates in plastics deconstruction processes and holistic gut community degradation processes, a collection of metaomics datasets is being developed. Synergized findings from transcriptomes, proteomes, and metabolomes, as well as hypothetical pathways for LDPE deconstruction, are iteratively being built. This community-wide systems biology approach allows for a complete picture of degradation processes by highlighting genes, proteins, and metabolites that coincide as upregulated in plastics-enriched gut communities.

Existing microbial isolates and those identified through metaomics approaches will ultimately be constructed into synthetic plastic-degrading communities capable of plastics waste valorization. To better recapitulate mealworm gut community behavior, researchers are investigating the ability of minimal synthetic microbial communities to deconstruct LDPE. Researchers identified cocultures that are metabolically active in media with LDPE as the sole carbon source. Preliminary data suggest that certain microbial isolates have enhanced plastics degradation potential in coculture conditions. The group is further investigating coculture behaviors using confocal microscopy to image microbial spatial variances and plastic particle surface colonization. Identifying essential features in minimal cocultures will inform the design of more complex synthetic communities capable of enhanced plastics degradation.

Beyond microbial work, researchers must consider polymer characteristics to develop improved deconstruction systems. Post-consumer waste plastics contain additive packages to improve processability, antioxidation, and flame retardancy. Given additive variability among polymer grades, researchers developed a standardized plastics preparation procedure wherein additives are stripped from polymers, leaving only the base plastic for deconstruction studies. The use of stripped plastics facilitates a more accurate comparison of deconstruction rates across plastic materials from various sources. Additionally, researchers' current work leverages successive self-seeding and annealing (SSA), differential scanning calorimetry (DSC), and thermal fractionation techniques to assess polymer architecture (e.g., branching densities) pre- and post-deconstruction. Preliminary data indicate that deconstruction predominantly occurs at low branching densities, indicating that high branching density plastics, such as LDPE, are less bioavailable than low branch-density plastics. Future efforts will concentrate on validating chain architecture hypotheses and elucidating the mechanism of polyethylene deconstruction from a branching perspective.

Funding Statement: This material is based upon work supported by the DOE Office of Science, BER program, GSP, under award number DE-SC0023085. A portion of this research will be performed under the Facilities Integrating Collaborations for User Science (FICUS) initiative and use resources at the DOE Joint Genome Institute (JGI) and the Environmental Molecular Sciences Laboratory (EMSL), which are DOE Office of Science User Facilities. Both facilities are sponsored by the BER program and operated under contract numbers DE-AC02-05CH11231 (JGI) and DE-AC05-76RL01830 (EMSL).

BioPoplar: a Tunable Chassis for Diversified Bioproduct Production

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Project Goals: This project will yield poplar chassis with multipurpose uses, including bioenergy, biomaterials, and bioproduct production. The generation of a robust cell type–specific set of transcription factors and cis-regulatory elements and the ability to modulate gene expression in a high-resolution manner (i.e., that of specific cell types) will enable precision genome engineering of metabolism, a significant advancement in capabilities in modulating plant biochemistry. The change in architecture will be exploited to permit production of bioproducts (e.g., drop-in fuel precursors in leaves), biomaterials (e.g., modified wood composition), and changes in agronomic production practices (e.g., increased stand density leading to increased yield). Collectively, these engineered chassis and tools provide the platform of a new era for poplar biology, agronomy, and processing.

Domestication and breeding efforts have shown that selection of specific plant architecture traits across a wide array of plant species, both annuals and perennials, results in improved traits for human use, either for food, feed, or fuel. Similarly, selective breeding can yield distinct chemotypes of crops with desired chemical profiles or compositions. Today, researchers can generate precision knowledge of gene regulation and function through high-resolution omics technologies and construct a synthetic biology toolkit to engineer plant genomes at DNA sequence, chromatin accessibility, and expression levels. Thus, science has entered an era where scientists can model, design, and then engineer precise changes in plant genomes that will lead to predictive, modified traits.

This project will re-engineer poplar as a multipurpose crop that can be used for bioenergy, biomaterial, and bioproduct production. The team will generate a cell atlas that encompasses gene expression, gene regulatory networks, and cis-regulatory elements responsible for gene expression at the cell-type level, providing the requisite knowledgebase and tools for precision biobased design and fabrication of multipurpose poplar. Researchers will couple single-cell datasets with new genome and epigenome editing tools to develop new morphotypes of poplar that have altered tree and leaf architecture. These morphotypes will substantially improve biomass potential via increased stand density, tree integrity, photosynthetic capture, and trichome density, and serve as the foundational chassis. These chassis will have altered ratios of leaves to stems and/or trichome density in which researchers can further engineer cell-wall composition and/or novel molecules (e.g., precursors for drop-in fuels), thus making poplar chemotypes that are "customized" to their biomaterial or bioproduct applications and simultaneously "maximized" in optimal morphotypes. The team will employ an iterative design process in which metabolic pathways are optimized to create unique chemotypes with tailored biomaterial and bioproduct composition.

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Single-Cell and Spatial Regulatory Map of Poplar

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Understanding the intricate regulatory mechanisms governing gene expression in plants is important for precise and efficient crop engineering. In this project, researchers present a comprehensive study aiming to construct a cell atlas elucidating the cis-regulatory elements (CREs) and the spatial gene expression network in poplar (*Populus* sp.). Leveraging a single-cell assay for transposase-accessible chromatin sequencing (scATAC-Seq), researchers dissect chromatin accessibility at the single-cell level to identify CREs related to important poplar traits. The team also applied spatial transcriptomics (spRNA-Seq) on various poplar tissues to unravel gene expression patterns in a spatial and temporal context. The resulting single-cell and spatial regulatory atlas will provide essential knowledge for re-engineering poplar as a multipurpose crop that can be used for bioenergy, biomaterial, and bioproduct production.

Knowledge-Guided Interrogation of the Plastid Fatty Acid Biofactory

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https://fatplants.net

Project Goals:

- Develop comparative systems datasets for typical (pennycress) versus extreme (*Cuphea*) seed fatty acid synthase (FAS).
- Develop a kinetic model for pennycress versus *Cuphea* FAS.
- Reconstitute and ratiometrically optimize FAS for increased fatty acid and C10 fatty acid production and kinetic model refinement.
- Generate synthetic biology–based FAS and acetyl-CoA carboxylase (ACCase) pennycress and *Camelina* biodesigns for increased oil and C10 fatty acid production.

Bigger, Better, Brassicaceae, Biofuels, and Bioproducts (B5) aims to dissect the biochemical underpinnings of fatty acid biosynthesis in plant plastids. This work will enable the predictable design of the nonfood Brassicaceae oilseeds *Camelina* and pennycress for increased seed oil and tailored fatty acid chain lengths to support U.S. bioenergy and oleochemical sectors. In oilseeds, energy-dense fatty acids are produced in plastids by type II FASs, which consist of discrete enzymes that function in concert to carry out the iterative elongation of fatty acid chains, two carbons at a time. FAS is fueled by malonyl-CoA generated by acetyl-CoA carboxylase (ACCase), the rate-limiting enzyme in fatty acid synthesis. Studies explore the hypothesis that the changes in the stoichiometry of both FAS enzymes and multimeric complexes of ACCase and associated regulatory proteins can enable fine-tuning of total fatty acid production and fatty acid chain lengths. The project's investigation of FAS composition and stoichiometry is informed by comparative analyses of seeds with a "typical" FAS (pennycress) that produces C16 and C18 fatty acids versus seeds with an "extreme" FAS (*Cuphea viscosissima*) that produces ~75% 10:0. As a first step, the team generated PacBio transcriptomes of developing pennycress and *C. viscosissima* seeds representing ~14,000 and ~21,000 gene families, respectively, which will be used for all integrative multiomics studies on data generated in this project. Through mining of these transcriptomes, researchers have generated a comprehensive set of cDNAs for FAS enzymes, ACCase enzymes, and regulatory proteins from seeds of both species. In pennycress seeds, researchers identified 37 genes, orthologous to *Arabidopsis*, involved in *de novo* fatty acid synthesis in the plastid. The team confirmed 31 key FAS and ACCase genes through global proteomic analysis of developing pennycress seeds. For absolute quantitation, 25 new absolute quantification (AQUA) peptides were synthesized alongside nine AQUA peptides shared with *Arabidopsis*. Researchers also expressed and purified recombinant pennycress FAS polypeptides, which will be used as standards in absolute quantitative proteomic studies and as constituents of *in vitro* FASs to support mathematical modeling of fatty acid synthesis. Early modeling efforts have focused on the integration of regulatory interactions associated with ACCase and FAS enzymes. To support the analysis of FAS compositions in developing seeds, the team also generated polyclonal antibodies against several recombinant FAS polypeptides that have yielded high-resolution western blots. Guided by prior research of the type II FAS of *Escherichia coli*, researchers are exploring the impact of changes in the expression of β-ketoacyl-acyl carrier protein synthase I (KASI), which acts as the initial condensing enzyme in fatty acid elongation, on yields of decanoic acid (C10) in pennycress and *Camelina* seeds engineered for the overproduction of this product. Overall, these collaborative efforts are generating fundamental knowledge that will be combined with B5 synthetic biology tool development for predictive design of optimized pennycress and *Camelina* feedstocks for biofuels and bioproducts.

Funding Statement: This research was supported by the DOE Office of Science, BER program, grant number DE-SC0023142.

Systems-Level Analysis of Extreme Differences in Fatty Acid Chain-Length Production: Natural Variants and Redesigned Brassicaceae Oilseeds

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Project Goals: The project addresses three goals: (1) systems-level analysis of *Camelina*, pennycress, and *Cuphea* for increased lipid content and predictable production of fatty acids with tailored chain lengths; (2) integration of a redesigned plastid biofactory with extra plastidial metabolism for enhanced oils within an engineered biocontainment strategy; and (3) controlled environment- and field-tested engineered germplasm.

Bigger, Better, Brassicaceae, Biofuels, and Bioproducts (B5) is providing fundamental knowledge to guide biodesigns of Brassicaceae nonfood oilseeds, *Camelina*, and pennycress for sustainable biofuels and bioproducts. One target is the tailoring of fatty acid biosynthesis and storage to generate *Camelina* and pennycress oils rich in medium-chain fatty acids (C8–C14) as feedstocks for sustainable aviation fuel. Researchers have undertaken a systems biology approach to understand the metabolic specialization that enables plants, such as *Cuphea* species, to accumulate oils highly enriched with medium-chain fatty acids versus typical oilseeds, such as *Camelina* and pennycress, that accumulate C16- and C18-rich oils. Researchers are also conducting a systems-level analysis of existing *Camelina* and pennycress lines engineered for C10 oil production to identify metabolic constraints that limit biosynthesis of these redesigned oils.

Lines are engineered with genes for further work through a design-build-test-learn strategy. Early transcriptomics results have been incorporated into 3D omics and CCMT tools for comparative and cross-species analytics. Results to date include measurements of biomass, metabolic intermediates, omics studies, and isotope tracer investigations.

Funding Statement: This research was supported by the DOE Office of Science, BER program, grant number DE-SC0023142.

Synthetic Biology Tool Development for Precision Engineering of Oilseed Crops

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https://fatplants.net/

Project Goals:

- Establish safe-harbor landing pads for predictable transgene expression.
- Characterize seed-specific promoters with a range of expression strengths.
- Define the impact of compositional genetic context effects on transgene expression with respect to alternative chromosomal loci for gene integration.
- Develop and use programmable transcriptional activators (PTAs) to regulate expression of multiple (endogenous) genes for seed oil manipulation.
- Target gene replacement at the native genomic locus in pennycress.

The random nature of *Agrobacterium*-mediated transgene insertion into plant genomes affects expression strength, resulting in unpredictable product accumulation and the need to characterize many independent transgenic lines. This greatly limits throughput of different gene combinations to efficiently explore the expression space needed for effective multi-gene pathway metabolic engineering. To overcome this limitation, researchers are creating a suite of tools for reliable engineering of multi-gene systems to provide predictable control of the level of transgene expression in *Camelina* and pennycress seeds. Specifically, this team will (1) generate *Camelina* and pennycress lines with safe-harbor landing pads, (2) develop a new set of seed-specific promoters with a range of expression strengths, and (3) leverage sequence-PTAs.

1. Safe-harbor landing pads: Researchers are developing C*amelina* and pennycress lines with safe-harbor landing pads that will use site-specific recombinase systems to enable the targeted insertion of transgenic cargos into predetermined genomic loci, allowing better control of transgene expression strength and reducing the number of independent transgenic lines to be characterized. Using a protoplast expression system, this group screened five recombinase systems for their effectiveness in pennycress. Two serine recombinases, Bxb1 and phiC31, possessed the highest recombination rate. Landing pads using the recombination sites of these two recombinases have been generated and transformed into both pennycress and *Camelina.*
2. Novel seed-specific promoters: The relative expression levels of genes involved in primary or secondary metabolism can dramatically affect product profile and titer. To augment the suite of very strong seed-specific promoters (e.g., napin, glycinin) currently used for oil seed engineering, the researchers have mined existing gene expression databases to identify nine seed-specific genes expressed at levels 1x, 0.1x, and 0.01x relative to the strong seed-specific promoters in these species (e.g., napin orthologs). Promoter and terminator sequences have been cloned for these genes to be compatible with the GoldenBraid cloning system and used to express two different lipid biosynthetic genes. Transgenic lines have been generated for all promoter/gene combinations and T2 seeds analyzed for the accumulation of target lipid molecules.
3. Sequence-PTAs: Catalytically deactivated Cas9 (dCas9 or dead Cas9) can be used as an RNA-guided DNA binding domain to deliver transcriptional activation or repression domains to a promoter of interest. These give unprecedented ability to control and fine-tune the expression level of multiple endogenous genes in parallel. Researchers have recently improved the performance of dCas9-based PTAs for plant applications by altering the mechanism by which activation domains are recruited to the dCas9 (Casas-Mollano et al. 2023) and by swapping out transcriptional activation domains derived from human viruses for those evolved in plant cells (Zinselmeier et al. 2022). Researchers now have transgenic lines of pennycress and *Camelina* that stably express the components of MoonTag PTAs and are in position to begin leveraging these tools for seed oil engineering.

Casas-Mollano, J. A., et al. 2023. "Efficient Gene Activation in Plants by the MoonTag Programmable Transcriptional Activator ," *NucleicAcids Research* **51**(13), 7083–93. DOI:10.1093/nar/gkad458.

Zinselmeier, M. H., et al. 2022. "Optimized dCas9 Programmable Transcription Activators for Plants," *bioRxiv*. DOI:10.1101/2022.06.10.495638.

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CRISPR-Cas Tool Development and Genome Engineering in Nonmodel Bacteria

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Project Goals: Develop a portable genetic toolkit for CRISPR-based gene regulation and genome engineering in nonmodel bacteria.

Nonmodel bacteria represent an untapped reservoir of metabolic diversity that could be harnessed to address urgent societal challenges, including sustainable bioproduction, climate change, plastic recycling, and environmental remediation. Metabolically versatile bacteria such as *Pseudomonas putida* and the purple nonsulfur bacteria *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* have emerged as chassis strains due to their ability to catabolize a variety of waste products and produce industrially relevant products. However, leveraging the capabilities of nonmodel microbes often requires the development of specialized genetic engineering and synthetic biology tools tailored to their unique genetic backgrounds. Thus, there is a need for portable genome engineering tools that can circumvent this bottleneck. The team is working to develop a portable toolbox for CRISPR-based approaches for gene regulation and genome engineering in nonmodel bacteria.

Multiomics-Driven Microbial Model Optimization

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https://sites.google.com/view/carothersresearchgroup/home

Project Goals: The goal is to create genome-scale models of endogenous metabolic pathways and develop metabolic sensitivity maps to identify reactions that dominate the control of flux. Specifically, researchers aim to gain insight into the regulatory mechanisms within pathways and predict outcomes of metabolic interventions at the genome scale.

Biomanufacturing poses a sustainable alternative to producing traditionally petrochemical-derived commodity chemicals. For these biomanufacturing efforts to be commercially viable, the design and engineering process must be targeted and quick. The advent of CRISPRa/i enables the targeted activation and repression of specific genes in organisms. Choosing which genes to target with CRISPRa/i for maximal yield through the metabolic pathway requires identifying which enzymes exert the most influence on the flux through the pathway (Kacser and Burns 1995). Bayesian Metabolic Control Analysis (BMCA) has been developed as a way to integrate omics data and genome-scale models to predict the flux control coefficients of a metabolic pathway (St. John et al. 2019). Yet, BMCA's predictive accuracy has yet to be quantified. Here, the results show that BMCA reliably predicts elasticity values in the absence of allosteric regulation and that the most informative type of data for BMCA is fluxomics, followed by enzyme concentrations, external metabolite concentrations, and internal metabolic concentrations. These results also demonstrate the fidelity and limitations of BMCA's predictions given the strength of CRISPRa/i perturbations in the dataset, and thereby establish guidelines for maximizing the predictive power of the BMCA method. This method was successfully applied to estimate sensitivities in random model topologies. Researchers anticipate that the insights drawn from this benchmarking study can be extended to other metabolic pathways, such as *Pseudomonas putida*.

Kacser, H., and J. A. Burns. 1995. "The Control of Flux," *Biochemical Society Transactions* **23**(2), 341–66. DOI:10.1042/bst0230341.

St. John, P. C., et al. 2019. "Bayesian Inference of Metabolic Kinetics from Genome-Scale Multiomics Data," *PLoS Computational Biology* **15**(11), e1007424. DOI:10.1371/journal.pcbi.1007424.

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Prototyping Carbon-Conserving Networks for Diacid Production

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Decarboxylation, the loss of carbon dioxide (CO_2) from a compound, is used in metabolism to commit carbon flux to a specific pathway. However, decarboxylation also limits the product carbon yield, with acetyl-CoA (two C_2) achieving only 66% carbon recovery when routing glucose (C_6) via glycolysis and oxidative decarboxylation. Carbon-conservation networks (CCNs) that circumvent CO_2 release can theoretically lead to carbon and product yields beyond those seen with endogenous metabolism (Westenberg and Peralta-Yahya 2023). Engineering metabolic pathways and developing technologies to improve carbon yield has the potential to increase the economic viability of large-volume low-cost chemicals. Toward this goal, the effects of overlaying CCNs have been mathematically modeled onto the endogenous metabolism of nonmodel organisms, such as *Pseudomonas putida* and *Rhodobacter sphaeroides*. The model predictions and prototyping combinations of existing and *de novo* CCNs predicted to improve carbon and product yields are now being implemented. As a proof of concept, the research team is measuring the effects of CCNs on the production of industrially relevant diacids: malic and itaconic. Going forward, the generality of CCNs will enable their implementation toward production of other large-scale chemicals that suffer from metabolic carbon loss.

Westenberg, R., and P. Peralta-Yahya. 2023. "Toward Implementation of Carbon-Conservation Networks in Nonmodel Organisms," *Current Opinion in Biotechnology* **81**, 102949. DOI:10.1016/j.copbio.2023.102949.

Stopping Escape and Malfunction in Genetic Code Engineered Cells

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Project Goals: The project engineered an ultra-safe strain of *Escherichia coli* for controlled growth and function, including production of peptides and proteins containing nonstandard amino acids.

Engineered cells can address unmet needs for planetary health. To develop safe cell-based technologies, researchers need engineering strategies that allow control of cellular proliferation and function. Currently, bacteria used as platform technologies rely on a wildtype genetic code, which can result in horizontal gene transfer, escape, and loss of control of the designed programs. Genetic code engineering emerges as a promising alternative since it removes a set of codons and tRNAs from the genome, which should prevent translation of incoming DNA. However, the team discovered a new mechanism of escape in bacteria with an engineered genetic code and characterized it with multiomics and protein language models, resulting in the development of an ultra-safe 61-codon *E. coli* strain. In this strain, researchers engineered a tRNA/aminoacyl-tRNA synthetase pair for the incorporation of a nonstandard amino acid and kill switches. This is the first organism that enables the production of proteins containing user-defined nonstandard amino acids while remaining tightly biocontained and bioisolated. This work is a headstart to developing ultra-safe living technologies and will allow researchers to decode and expand genome and protein designs.

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Rapid Discovery and Evolution of Nanosensors Containing Fluorogenic Amino Acids

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Binding-activated optical biosensors are powerful tools for imaging, diagnostics, and biomolecule sensing. However, the discovery of new biosensors with current approaches is slow, requiring tedious rational design, empirical screening, and characterization. Here, researchers report a platform that streamlines biosensor discovery, production, and characterization and unlocks directed biosensor evolution with genetically encodable fluorogenic amino acids (FgAAs). The team first improved on the classical semisynthetic approach by engineering <15 kilodalton "nanosensors" that specifically recognize proteins, peptides, and small molecules with up to 100-fold fluorescence increases and subsecond kinetics, allowing real-time and wash-free target sensing and live-cell bioimaging. Next, the site-specific incorporation of new FgAAs by an optimized genetic code expansion chemistry enabled cell-free, genetic translation of functional nanosensors and benchmarking of hundreds of nanosensor candidates in parallel. This capability allowed the team to identify improved nanosensors by an unbiased and rapid (~3 hour) ribosomal discovery approach and establish methods of directed nanosensor evolution and machine learning (ML)-guided nanosensor optimization. The directed nanosensor evolution pipeline enabled researchers to select for nanosensors with switched target specificity or dramatically improved sensitivities (up to ~250-fold). Once trained on the current FgAA-containing nanosensor data, researchers expect the ML algorithms will facilitate nanosensor engineering both with novel FgAAs as well as against new targets. Ultimately, the team envisions the pipeline will be applicable to diverse proteins containing different nonstandard functionalities. Altogether, this synthetic biology platform will accelerate the discovery of biosensors and be broadened further to augment proteins with other nonstandard amino acid functionalities for new applications.

Construction of a Synthetic 57-Codon *E. coli* Chromosome to Achieve Resistance to All Natural Viruses, Prevent Horizontal Gene Transfer, and Enable Biocontainment

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https://arep.med.harvard.edu

Project Goals: The research group is finalizing the construction of a fully recoded, 3.97 megabase pair *Escherichia coli* genome that relies on the use of only 57 genetic codons. For this aim, the genome was computationally designed, synthesized, and assembled into 88 segments. In the final steps of genome construction, researchers combine and optimize these segments *in vivo* to assemble the fully recoded, viable chromosome. In parallel with the construction of this 57-codon organism, the team is investigating how mobile genetic elements and environmental viruses overcome the genetic isolation of organisms bearing modified genetic codes.

Researchers present the construction of a recoded, 57-codon *E. coli* genome, in which seven codons are replaced with synonymous alternatives in all protein-coding genes. For this aim, the entirely synthetic recoded genome was assembled as 88, 21 to 52 kilobase-pair episomal segments, individually tested for functionality, and then integrated into the genome. Developing a specialized integration system and optimizing the team's workflow enhanced integration efficiency to 100%, resulting in an order of magnitude increase in construction speed. The team is now combining recoded genomic clusters with a novel technology that builds on the group's latest developments in recombineering and CRISPR-associated nucleases (Wannier et al. 2020; Wannier et al. 2021). In parallel with genome construction, researchers developed novel experimental methods to identify fitness-decreasing changes and troubleshoot these cases. Leveraging massively parallel genome editing and accelerated laboratory evolution allowed the group to correct partially recoded strains' fitness within weeks (Nyerges et al. 2018). As researchers approach the final assembly of this *E. coli* genome, they also implement dependency on nonstandard amino acids.

The team's previous experiments showed that rational genetic code engineering could isolate genetically modified organisms (GMOs) from natural ecosystems by providing resistance to viral infections and blocking horizontal gene transfer (HGT); however, how natural mobile genetic elements and viruses could cross this genetic code–based barrier remained unanswered. By systematically investigating HGT into *E. coli* Syn61Δ3, an *E. coli* strain with a synthetic 61-codon genetic code, the group discovered that transfer (t) RNAs expressed by viruses and other mobile genetic elements readily substitute cellular tRNAs and abolish genetic-code-based resistance to HGT (Nyerges et al. 2023). Researchers also discovered 12 new bacteriophages in environmental samples that can infect and lyse this 61- codon organism. These viruses express 10 to 27 tRNAs, including functional tRNAs needed to replace the host's missing tRNA genes. The team also identified viruses with tRNAs that hold the potential to abolish the virus resistance of this 57-codon organism. These findings suggest that the selection pressure of organisms with compressed genetic codes can facilitate the rapid evolution of viruses and mobile genetic elements capable of crossing a genetic-code-based barrier. Therefore, researchers developed additional genetic biocontainment technologies to simultaneously block GMOs' unwanted proliferation, eliminate viral infections, and prevent transgene escape (Nyerges et al. 2023).

In sum, this research group's genome synthesis work will soon (1) demonstrate the first 57-codon organism; (2) establish a tightly biocontained chassis for new-to-nature protein production; and (3) open a new avenue for the bottom-up synthesis and refactoring of microbial genomes, both computationally and experimentally. Furthermore, the researchers demonstrate that horizontally transferred tRNA genes of mobile genetic elements and viruses can substitute deleted cellular tRNAs and thus rapidly abolish compressed genetic codes' resistance to viral infections and HGT.

Nyerges, A., et al. 2018. "Directed Evolution of Multiple Genomic Loci Allows the Prediction of Antibiotic Resistance," *Proceedings of the National Academy of Sciences* **115**(25), E5726–35. DOI:10.1073/pnas.1801646115.

Nyerges, A., et al. 2023. "Swapped Genetic Code Blocks Viral Infections and Gene Transfer," *Nature* **615**, 720–7. DOI:10.1101/2022.07.08.499367.

Wannier, T. M., et al. 2020. "Improved Bacterial Recombineering by Parallelized Protein Discovery," *Proceedings of the National Academy of Sciences*. DOI:10.1073/pnas.2001588117

Wannier, T. M., et al. 2021. "Recombineering and MAGE," *Nature Reviews Methods Primers* **1**(1), 1–24. DOI:10.1038/s43586-020-00006-x.

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Cryosectioning-Enabled Super-Resolution Microscopy for Studying Nuclear Architecture at the Single-Protein Level

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DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) combined with total internal reflection fluorescence (TIRF) microscopy enables the highest localization precisions, down to single nanometers in thin biological samples. However, most cellular targets, including the nucleus, elude the accessible TIRF range close to the coverglass and thus require alternative imaging conditions, affecting resolution and image quality. Here, researchers address this limitation by applying ultrathin physical cryosectioning in combination with DNA-PAINT. With tomographic and kinetically enhanced DNA-PAINT (tokPAINT; Stein et al. 2024), the group demonstrates imaging of nuclear proteins with sub-3-nanometer localization precision, advancing the study of nuclear organization within fixed cells and mouse tissues at the level of single antibodies. This team believes ultrathin sectioning combined with the versatility and multiplexing capabilities of DNA-PAINT should be able to contribute to the study of proteins, RNA, and DNA in genome organization at the molecular level and *in situ*.

Stein, J., et al. 2024. "Cryosectioning-Enabled Super-Resolution Microscopy for Studying Nuclear Architecture at the Single Protein Level," *bioRxiv*. DOI:10.1101/2024.02.05.576943.

Novel Systems Approach for Rational Engineering of Robust Microbial Metabolic Pathways

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The goal of this project is to develop and implement a process for improving bioproduction under conditions that are appealing for industrial processes, such as high temperature and low pH. The approach addresses the failure of metabolic reactions due to inhibition, denaturation, misfolding, or disorder of enzymes. Researchers have developed and implemented a framework for identifying these enzymes and selecting robust replacement enzymes, using high temperature and low pH as model stressors in *Escherichia coli*. The engineering strategy of replacing enzymes to improve bioproduction is well-established but rarely applied to system-wide stressors. This approach is complementary to improvement of microbial robustness by engineering the cell membrane and has advantages relative to evolutionary-based organism improvement by prioritizing bioproduction rather than growth.

Temperature Sensitivity: There is a wealth of data available regarding enzyme structural integrity at high temperatures. Researchers are using an *in vitro* metabolomics approach for proteome-wide analysis of enzyme activity at high temperatures. Candidate bottleneck enzymes have been identified and investigated, including homoserine O-succinyltransferase (MetA), biotin synthase (BioB), ketol-acid reductoisomerase (IlvC), and 3-oxoacyl-ACP synthase 1 (FabB). For these candidate enzymes, sequences from thousands of various microorganisms with differing temperature ranges of growth have been collected and aligned. Experimental and predicted structures of these enzymes are used to query the enzyme dynamics, with the goal of identifying which sequence differences account for the ability of these critical enzymes to function at elevated temperatures.

Acid Sensitivity: The pH tolerance efforts have prioritized modeling the effect of pH on the allocation of cellular resources. The metabolic model accounts for the effect of intracellular acidification on cellular energetics, the thermodynamic characteristics of metabolic reactions, and

enzyme activity. Each of these three model adjustments contribute to the predicted flux distribution. A sensitivity analysis is in progress to identify the most critical enzymes for replacement. Borrowing from the abundant proteomic data of enzyme stability in the presence of increasing temperatures, researchers have developed a proteomic approach for enzymes with structural sensitivity to decreasing pH. This approach has identified several enzymes critical for central metabolism with poor acid tolerance. Researchers are also using models of enzyme temperature sensitivity as inspiration in the development of predictive sequence- and structure-based models of enzyme pH sensitivity.

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Using Cell-Free Systems to Accelerate Biosystems Design for Carbon-Negative Manufacturing

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The accelerating climate crisis combined with rapid population growth poses some of the most urgent challenges to humankind, all linked to the unabated release and accumulation of carbon dioxide (CO_2) across the biosphere. By harnessing the capacity to partner with biology, the abundance of available CO_2 can be leveraged to transform the way the world produces and uses carbon. Yet, designing, building, and optimizing nonmodel CO_2-fixing biosystems to achieve a broader range and more complex biofuels, bioproducts, and biomaterials remains a formidable challenge.

To address this challenge, the research team is developing a cell-free protein synthesis approach for high-throughput engineering of natural and novel enzymes for CO_2 assimilation and biosynthetic product pathways. In one example, the team uses cell-free systems to study natural enzymes like ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which is the key enzyme of the Calvin cycle. Various types of RuBisCO were successfully expressed, including form I and form II, via cell-free protein synthesis, and their activity was confirmed by NADH-linked assay and liquid chromatography–mass spectroscopy.

In another example, hydroxyacyl-CoA lyases (HACLs) are being engineered. HACLs have become increasingly relevant due to their ability to form carbon-carbon bonds between formyl-CoA (C_1 donor) and a larger carbonyl-containing molecule (C_1 acceptor). The research team has expressed, purified, and characterized over 60 homologs selected by sequence similarity, uncovering the high promiscuity of these enzymes.

Collectively, these efforts demonstrate how cell-free systems can be used as a screening tool to explore the wide scope of natural enzyme diversity. The newly characterized enzymes can contribute to the engineering of C_1 assimilation routes and expand branches of synthetic metabolism to enable a diverse set of enzymatic reactions for sustainable bioproduction.

Engineering *Cupriavidus necator* for Efficient Aerobic Conversion of Carbon Dioxide to Fuels and Chemicals

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Project Goals: This project broadly aims to establish platforms for *in vivo* and *in vitro* conversion of carbon dioxide (CO_2) to fuels and chemicals through the advancement of genetic engineering and predictive biosystems design tools.

The ecological and societal consequences of anthropogenic climate change necessitate the transition away from fossil fuels as a primary source of energy and chemical products. Biological processes utilizing microbes as a platform for synthesizing fuels and chemicals from ubiquitous, renewable carbon sources such as CO_2 provide an opportunity to replace traditional industrial processes with sustainable, carbon-negative biomanufacturing. However, engineering chassis microbes to convert CO_2 to product efficiently in scaled-up industrial bioprocesses remains challenging. This project broadly aims to establish platforms for *in vivo* and *in vitro* conversion of CO_2 to fuels and chemicals through the advancement of genetic engineering and predictive biosystems design tools. At NREL, researchers are focused on (1) developing new machine learning tools for enzyme engineering and strategies for evaluating enzyme activity and (2) improving the ability of the industrial host

Cupriavidus necator to assimilate CO_2 and produce fuel and nylon precursor molecules aerobically. The team conducts ongoing work establishing methods for screening native and engineered enzyme activity and efforts to engineer *C. necator* for efficient CO_2 conversion. *C. necator* has a highly versatile and robust metabolism and can grow to high cell densities on a variety of carbon and energy sources, including CO_2 and H_2 as the sole source of carbon and energy. Researchers focus efforts on enabling *C. necator* to produce high titers of β-ketoadipate (βKA), a dicarboxylic acid that can be incorporated into high-performance nylons, and the terpenoids myrcene and bisabolene, which are precursors for sustainable aviation fuel and diesel blendstocks. Researchers work to identify which of the 70+ putative βKA-degradation enzymes in the *C. necator* genome are active in degrading βKA, and report progress on deleting these enzymes to engineer a strain that accumulates high amounts of βKA. Additionally, researchers work towards enabling high-titer terpenoid production via the addition of a heterologous synthesis pathway. Finally, researchers work to streamline the genome of *C. necator* for efficient autotrophic growth in a bioreactor environment. Overall, this work will result in next-generation tools for biosystems design and advance *C. necator* as an industrial chassis for conversion of CO_2 to value-added products in carbon-negative manufacturing processes.

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Accelerating Carbon-Negative Biomanufacturing Through Systems-Level Biology and Genome Optimization

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Project Goals: To develop high-throughput biosystems design tools that are applied to multiple testbeds for carbon-negative biomanufacturing.

In the face of escalating climate change, there is a pressing need for innovative strategies to mitigate carbon emissions. LanzaTech stands at the forefront of this initiative, employing chemoautotrophic gas fermenting microorganisms to convert carbon dioxide (CO_2) into valuable C-based materials. This multidisciplinary project aims to enhance the metabolic efficiency of CO_2-utilizing biosystems through genome optimization and the integration of machine learning (ML) techniques. This approach employs cutting-edge genomic tools to iteratively knock out gene clusters towards engineering strains streamlined for the rigorous conditions of industrial fermentation. Given the abundant possible permutations, researchers leverage ML to inform the experimental strategies and strategically guide the knockout efforts. To this end, researchers have trained ML models using transcriptomic datasets to discern intricate patterns and relationships between genes and their functions. These models will be leveraged towards identifying contiguous genetic regions for targeted reduction. Strains generated from this process will not only enhance the understanding of gene functionality, they also enable the construction of genotype–phenotype associations through downstream screening (Sastry et al. 2019; Sastry et al. 2021). These efforts will significantly advance *in silico* models and streamline the development of microbial strains for industrial-scale applications.

Sastry, A.V., et al. 2019. "The *Escherichia coli* Transcriptome Mostly Consists of Independently Regulated Modules," *Nature Communications* **10**, 5536. DOI:10.1038/s41467-019-13483-w.

Sastry, A.V., et al. 2021. "Independent Component Analysis Recovers Consistent Regulatory Signals from Disparate Datasets," *PLOS Computational Biology* **17**(2), e1008647. DOI:10.1371/journal.pcbi.1008647.

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Engineering a Carbon Dioxide Concentrating Mechanism in *Cupriavidus necator* for Carbon-Negative Biomanufacturing

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Project Goals: The program goal is to develop high-throughput biosystems design tools in carbon dioxide (CO_2)-fixing biosystems and apply these tools to engineer biosynthetic pathways for carbon-negative biomanufacturing of simple commodity chemicals. In this project, researchers aim to increase the CO_2 utilization efficiency of the CO_2-fixing microbe, *Cupriavidus necator,* by reconstituting the α-carboxysomal CO_2-concentrating mechanism (CCM) native to *Halothiobacillus neapolitanus.*

The accelerating climate crisis combined with rapid population growth poses some of the most urgent challenges to humankind. A major contributing factor to this crisis is the unabated release and accumulation of CO_2 across the biosphere. Researchers can take advantage of this abundance of available CO_2 to transform the way the world produces and uses carbon by engineering CO_2-fixing biosystems to produce commodity fuels and chemicals. A CO_2-fixing organism that is actively being studied as a C-negative biomanufacturing chassis is the bacterium *C. necator.* While proficient in producing high titers of metabolic products, wildtype *C. necator* does not grow optimally at ambient levels of CO_2 in comparison to at high CO_2 conditions on autotrophic metabolism. Researchers propose to optimize *C. necator* growth under atmospheric conditions by heterologously expressing the α-carboxysomal CCM characterized in *H. neapolitanus* (Desmarais et al. 2019; Flamholz et al. 2020). To enable stable chromosomal expression of this 20kb *H. neapolitanus* CCM operon in *C. necator*, the team developed an inducible landing pad for integrase-mediated integration of synthetic cargo. In this project, the team demonstrates integration of the bacterial luminescence pathway and tunable expression of the pathway from the landing pad in *C. necator*. Another objective is to encapsulate the native *C. necator* RuBisCO into the carboxysomal structures instead of heterologously expressing the *H. neapolitanus* RuBisCO. *C. necator* RuBisCO has been reported to retain optimal carboxylation rate in aerobic conditions with abundant competing O_2, which is an advantageous trait to maintain for aerobic cultivation (Satagopan and Tabita 2016). Researchers have utilized RFdiffusion, a protein-design software, to *de novo* design *C. necator* RuBisCO binding motifs to replace with the *H. neapolitanus* RuBisCO for carboxysomal encapsulation (Watson et al. 2023). The next steps will entail synthesizing and testing these motifs *in vitro* for binding.

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Watson, J. L., et al. 2023. "*De Novo* Design of Protein Structure and Function with RFdiffusion," *Nature* **620**. DOI:10.1038/s41586-023-06415-8.

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Developing, Understanding, and Harnessing Modular Carbon/Nitrogen-Fixing Tripartite Microbial Consortia for Versatile Production of Biofuel and Platform Chemicals

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Project Goals: The overall goal of this project is to design, construct, analyze, and optimize a synthetic microbial consortium system consisting of three closely interacting members—a carbon dioxide (CO_2)-fixing photosynthetic specialist, a nitrogen (N_2)-fixing specialist, and a third specialist that can convert organic carbon (C) and N generated by the first two specialists to synthesize a desired product. By integrating complimentary expertise from multiple research laboratories at three institutions, researchers are pursuing three specific objectives: (1) develop tripartite microbial consortia for C or N fixation and production of biomolecules with various N or C ratios; (2) investigate molecular and cellular mechanisms governing the tripartite consortia via omics study and predictive modeling; and (3) explore alternative spatial configurations and develop scalable design principles.

Microbial communities are ubiquitous in nature, exhibiting incredibly versatile metabolic capabilities and remarkable robustness. Inspired by these synergistic microbial ecosystems, rationally designed synthetic microbial consortia is emerging as a new paradigm for bioprocessing and offers tremendous potential for solving some of the biggest challenges the society faces. In this project, researchers focus on a tripartite consortium consisting of a CO_2-fixing photosynthetic specialist, a N_2-fixing specialist, and a third specialist that can convert organic C and N generated by the first two specialists to synthesize a desired product. In addition to CO_2 fixation, a noteworthy feature of this design is the elimination of the requirement for N fertilizer, which has been produced through ammonia synthesis using the Haber-Bosch process and accounts for an estimated 2% of global energy expenditure. Researchers aim to develop a modular and flexible model system capable of producing diverse biomolecules (varying C:N ratio) as advanced biofuel or

platform chemicals, to dissect this complex ecosystem using a spectrum of cutting-edge systems approaches, and to ultimately derive scalable and broadly applicable design principles for maximizing the system performance.

The team's first prototype tripartite consortium employs genetically modified strains of photosynthetic cyanobacterium *Synechococcus elongatus* that secretes sucrose and N-fixing bacterium *Azotobacter vinelandii* that secretes ammonia to form a symbiotic foundation for supporting a third producer member (Abramson et al. 2016; Barney et al. 2015). Utilizing a customized bioreactor system consisting of multiple chambers separated with permeable membranes, and allowing control of growth rates of individual consortium members, researchers demonstrate this platform technology with selected representative production specialists, including a sucrose-metabolizing *Escherichia coli* K-12 derivative strain, *Corynebacterium glutamicum*, and *Bacillus subtilis* (Carruthers et al. 2020; Carruthers et al. 2024).

The team's ongoing work aims to develop new methods for creating novel spatial configurations that provide individualized environmental niches for each consortium member and thereby maximize their performance on intended functionalities. One initial focus is to dissect spatially separated and spatially consolidated cultivations of *Azotobacter vinelandii* and *Synechococcus elongatus*. Omics studies are conducted to unravel regulatory mechanisms. This allows the ability to gain fundamental insights on species interactions and their contributions to the robustness of the biculture system, which will guide future efforts in optimization of the whole system.

Other ongoing work includes: (1) development of predictive mathematical models to systematically explore the parameter space to understand how different biological parameters and operating strategies impact the system performance such as yield and productivity; and (2) investigation of spatially organized cocultures using 3D-printed communities in hydrogel matrices, which render high-resolution control and analysis capabilities.

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Carruthers, D., et al. 2020. "Random Chromosomal Integration and Screening Yields *E. Coli* K-12 Derivatives Capable of Efficient Sucrose Utilization," *ACS Synthetic Biology* **9**(12), 3311–21.

Carruthers, D., et al. To be submitted 2024. "Engineering Modular Carbon- and Nitrogen-Fixing Microbial Consortia for Sustainable Biochemical Production."

Funding Statement: This research was supported by the DOE Office of Science, BER program, grant number DE-SC0022136.

A Systems Approach for Predicting Metabolic Fluxes in *Auxenochlorella protothecoides*

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Project Goals: *Auxenochlorella protothecoides*, a Trebouxiophyte oleaginous alga, is a reference for discovery and a platform for photosynthesis-driven synthetic biology and sustainable bioproduction. The project will expand transformation markers, regulatory sequences, and reporter genes; improve transformation efficiency; and develop ribonucleoprotein-mediated gene-editing methods for genome modification. Systems analyses and metabolic modeling approaches will inform genome modifications for rational improvement of photosynthetic carbon fixation and strain engineering to produce cyclopropane fatty acids. Regulatory factors and signaling pathways responsible for activating fatty acid and triacylglycerol biosynthesis will be identified, and the team will manipulate them to increase lipid productivity. Nonphotochemical quenching and a regulatory circuit for maintaining photosynthesis under copper limitation, both of which are absent in *A. protothecoides*, will be introduced to improve photosynthetic resilience, and the performance of engineered strains will be modeled.

One approach to inform the design of production strains is the use of metabolic models to identify novel gene targets and reduce the potential solution space to maximize productivity. Using a high-quality genome sequence and highly accurate annotations, researchers have generated a complete metabolic network of *A. protothecoides*. To have representative biomass formation equations for a variety of growth conditions, the team has measured the macromolecule content and composition of *A. protothecoides* in autotrophic, mixotrophic, and heterotrophic growth regimes. This data, coupled with experimentally determined uptake and excretion rates, was used to constrain the model. Researchers simulated growth in the different growth regimes and performed a gene knockout analysis to determine gene essentiality and the impact of knockouts on fatty acid production. The complete genome scale model and the simulation results will be presented.

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Tunable Leaky Ribosomal Scanning Governs Translation of Polycistronic Genes in Green Algae

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Project Goals: Facile gene targeting in the nuclear genome of *Auxenochlorella protothecoides*, a unicellular, freshwater Trebouxiophyte, make this a useful reference organism for discovery and a platform for synthetic biology. Researchers aim to expand the molecular genetic toolkit with additional neutral integration sites, transformation markers, regulatory sequences, and reporter genes, along with improving transformation efficiency and developing RNP-mediated gene-editing methods for genome modification. Researchers are employing systems analyses and metabolic modeling approaches to inform engineering of the Calvin-Benson cycle for improved photosynthetic carbon fixation, and to identify signaling pathways and regulators responsible for controlling fatty acid and triacylglycerol biosynthesis. Genome modifications predicted from these analyses to increase lipid productivity will be combined with strain engineering to produce cyclopropane fatty acids. Non-photochemical quenching and a regulatory circuit for maintaining photosynthesis under copper-limitation, both of which are absent in *A. protothecoides*, will be introduced to improve photosynthetic resilience.

Polycistronic genes encode two or more polypeptides on a single mRNA molecule. In prokaryotic, mitochondrial, and plastid genomes, open reading frames (ORFs) are usually clustered via function, and transcription and translation of polycistronic transcripts are coupled. In eukaryotes, canonical gene expression is generally considered exclusively monocistronic since the cap-dependent scanning mechanism of translation initiation limits recognition of multiple ORFs. However, advances in sequencing technology have revealed cases of polycistronic mRNAs in various plants, animals, and fungi. In the process of updating genome annotations for two green algae, *Chlamydomonas reinhardtii* and *Chromochloris zofingensis*, long-read Iso-seq sequencing revealed an abundance of polycistronic genes, though the mechanism controlling the synthesis of proteins remained elusive (Gallaher et al 2021). *A. protothecoides,* an oleaginous Trebouxiophyte that is evolutionarily divergent from both *C. reinhardtii* and *C. zofingensis*, is a valuable reference organism for molecular genetics and biotechnology. While assembling and annotating the *Auxenochlorella* nuclear genome, researchers discovered a new set of conserved polycistronic genes. The team also updated the inventory of polycistronic genes in the genome of *C. reinhardtii*, using available Ribo-seq data to strengthen the assignments, and established high-confidence polycistronic gene datasets from both *C. reinhardtii* and *A. protothecoides* for investigation. Examination of the structural features of bicistronic genes revealed preferences for short 5' untranslated regions, weak Kozak sequences for the first ORF, and bias against alternative ATG translation start codons in all regions upstream of the second ORF. A*uxenochlorella* endogenous polycistronic loci were cloned and integrated at a neutral locus by homologous recombination, and the second ORF was swapped with a Venus fluorescent reporter gene. Manipulation of the ORF1 Kozak sequence altered the expression of the Venus reporter, with a weaker Kozak sequence conferring greater expression and a stronger Kozak sequence decreasing expression. Removal of the ORF1 start codon substantially increased ORF2 expression. A synthetic polycistronic dual reporter showed inversely adjustable activity of green fluorescent protein expressed from ORF1 and luciferase from ORF2, depending on the ORF1 Kozak strength. The results demonstrate that expression of multiple ORFs in green algal polycistronic transcripts occurs by means of alternative translation initiation and are consistent with leaky ribosome scanning as the most probable mechanism. The design logic behind these polycistronic genes will be implemented in future metabolic engineering projects to co-express and precisely control the stoichiometry of proteins produced by transgenes. In addition, researchers will undertake functional analysis of enigmatic endogenous polycistronic loci.

Gallaher, S. D., et al. 2021. "Widespread Polycistronic Gene Expression in Green Algae," *Proceedings of the National Academy of Sciences* **118**, e2017714118.

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Engineering *Auxenochlorella protothecoides*: Artificial Chromosomes, Regulators of Lipid Biosynthesis, and Improving Photosynthesis

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Project Goals: Facile gene targeting in the nuclear genome makes *Auxenochlorella protothecoides*, a unicellular, freshwater Trebouxiophyte, useful as a reference organism for discovery and a platform for synthetic biology. The team aims to expand the molecular genetic toolkit with additional neutral integration sites, transformation markers, regulatory sequences and reporter genes, along with improving transformation efficiency and developing RNP-mediated gene-editing methods for genome modification. Researchers are employing systems analyses and metabolic modeling approaches to inform engineering of the Calvin-Benson cycle for improved photosynthetic carbon (C) fixation, and to identify signaling pathways and regulators responsible for controlling fatty acid and triacylglycerol biosynthesis. Genome modifications predicted from these analyses to increase lipid productivity will be combined with strain engineering to produce cyclopropane fatty acids. Nonphotochemical quenching and a regulatory circuit for maintaining photosynthesis under copper limitation, both of which are absent in *A. protothecoides*, will be introduced to improve photosynthetic resilience.

Researchers used PacBio long-read sequencing to generate a gapless, telomere-to-telomere, phased diploid nuclear genome, and fully resolved the circular organelle genomes of *A. protothecoides* UTEX 250. This well-annotated, 45 Mb diploid nuclear genome resembles a genetic hybrid, with extensive inter- and intrachromosomal recombination and two instances of trisomy. Chromosome 3 trisomy was confirmed by knock-in of a Venus reporter at one allele of ammonium transporter 1B (*AMT1B*), and activation of the *AMT1B* promoter by nitrogen depletion in heterotrophic cells resulted in increased Venus fluorescence. The team will exploit this redundant chromosome as a landing pad for transgene integration, and putative centromere sequences will be tested for their ability to allow maintenance of stable centromeric plasmids.

Photosynthgreen algae can utilize sunlight to power photosystems for C fixation. In the daytime, algae are exposed to dynamic light conditions ranging from high-to-low or dark. Changing light conditions have significant impacts on their growth, biomass, and production. Researchers propose two strategies to improve C capture and growth under a field-like setting for *A. protothecoides*: (1) engineering a rate-limiting enzyme of the Calvin-Benson cycle (CBC), sedoheptulose 1,7-bisphosphatase (SBPase); and (2) introducing a photoprotective nonphotochemical quenching (NPQ) protein (LHCSR) to allow for robust growth under fluctuating light. Taking advantage of homologous recombination, researchers have generated strains to test both strategies. Preliminary data indicate that overexpression of SBPase leads to a growth benefit, and the introduction of a well-characterized algal LHCSR improves NPQ kinetics. Molecular characterization of the engineered strains is in progress to understand the changes in the metabolic flux through CBC and the regulation of the newly introduced NPQ and to determine whether these modifications confer growth advantages under dynamic light conditions.

Transcription factors play critical roles in transcriptional regulation of fatty acid biosynthesis (FAS) genes and can be used in genetic engineering approaches to increase the expression of the FAS pathway. Researchers established a pipeline to extract transcription factors based on InterProScan identifiers from the *A. protothecoides* UTEX 250 genome. In parallel, the team conducted a proteomics experiment under lipid-accumulating conditions (nitrogen depletion and glucose addition). These analyses identified novel putative transcription factors that are upregulated in lipid-accumulating cells and therefore are candidates for involvement in regulation of acclimation to nitrogen starvation, glycolysis, and *de novo* fatty acid synthesis. Researchers are currently generating mutants to test the roles of these candidate transcription factors. Altogether, this work will inform the engineering of strains to increase total lipid accumulation in *A. protothecoides*.

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Metabolic Remodeling: Stylish Options for Bacterial Interior Design

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Project Goals: This project seeks to expand the metabolic capabilities of a genetically malleable soil bacterium, *Acinetobacter baylyi* ADP1. This strain naturally degrades a wide variety of plant-derived aromatic compounds.

Augmentation and alteration of these natural capabilities have the exciting potential to improve biotechnology applications ranging from lignin valorization to biomanufacturing. This team's specific aims are to create novel pathways for the catabolism of syringol and pyrogallol and to develop new methods for large-scale genomic remodeling.

Advances in bacterial metabolic engineering and synthetic biology enable comprehensive genomic change. Altered aromatic compound metabolism in *Acinetobacter baylyi* ADP1 is facilitated by its exceptionally efficient natural transformation system. In this project, success was achieved by combining multiple approaches including enzyme design and the construction of modular synthetic pathways. Nevertheless, the biological consequences of such manipulation are often unpredictable. To achieve desired results, a growth-based adaptive evolution method was developed, called Evolution by Amplification and Synthetic biology (EASy) (Tumen-Velasquez 2018). With this method, the targeted amplification of chromosomal regions serves as a rudimentary form of regulation to balance expression of different pathway segments.

Researchers focused on creating a pathway for syringol (2,6-dimethoxyphenol) degradation. This compound arises during lignin pyrolysis from the decomposition of sinapyl alcohol moieties. When converting lignin-derived mixtures to valuable products by microbes, syringol can be problematic both as an inhibitory compound and as an underutilized substrate. Since there is no characterized pathway for syringol consumption, researchers designed one to be expressed from the *A. baylyi* chromosome. In general, aromatic compound catabolism can be considered modular. The first module involves reactions to generate one of a limited number of aromatic substrates of ring-cleavage enzymes. The next key step is ring-cleavage itself, accomplished aerobically using *ortho* (intradiol) or *meta* (extradiol) dioxygenases. Finally, a multistep "lower" pathway typically feeds metabolites to central metabolism.

Researchers sought to convert syringol to pyrogallol, a potential ring-cleavage target. Pyrogallol cleavage can be mediated by some *ortho*- and *meta*-catechol dioxygenases, although specific protein sequences and responsible enzymes remain unknown. Strains were constructed to express combinations of different catechol dioxygenases and a guaiacol demethylase (GcoAB) variant, which converts syringol to pyrogallol (Machovina et al. 2019). The team's initial attempts failed to express two separate enzymes capable of producing and cleaving pyrogallol from syringol. In contrast, colorometric assays suggested that the rational design of a novel chimeric enzyme successfully led to the *in vivo* metabolism of syringol and to the cleavage of pyrogallol by *A. baylyi* cells. The design of this chimeric enzyme was based on a similar enzyme that emerged from EASy experiments using guaiacol as a growth substrate (Tumen-Velasquez 2018). Key to success was the choice of sequence encoding an *ortho*-cleaving catechol dioxygenase with augmented activity on pyrogallol compared to the native version of this enzyme (CatA). Thus, these steps, mediated by a fabricated enzyme, represent two modules of a synthetic pathway for syringol degradation. As the final module to enable syringol to be used as growth substrate, the team incorporated a foreign pathway that is not native to *A. baylyi* for protocatechuate metabolism. Researchers mixed and matched these modules for functionality using a variety of different aromatic growth substrates. Growth on aromatic substrates using these nonnative pathways involved targeted gene amplification and combinations of mutations selected during laboratory evolution. Collectively, these results highlight the feasibility of large-scale genomic remodeling for biotechnology. ADP1 offers exciting potential for further development as a synthetic biology chassis.

Machovina M. M, et al. 2019. "Enabling Microbial Syringol Conversion Through Structure-Guided Protein Engineering," *Proceedings of the National Academy of Sciences of the U.S.A.***116**, 13970–76. DOI:10.1073/pnas.1820001116.

Tumen-Velasquez, M., et al. 2018. "Accelerating Pathway Evolution by Increasing the Gene Dosage of Chromosomal Segments," *Proceedings of the National Academy of Sciences of the U.S.A.* **115**, 7105–10. DOI:10.1073/pnas.1803745115.

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Engineering Synthetic Anaerobic Consortia Inspired by the Rumen for Biomass Breakdown and Conversion

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Project Goals: This project will leverage a synthetic rumen consortium composed of anaerobic fungi and chain-elongating bacteria to study which metabolites are shared and exchanged between microbes and identify strategies to bolster lignocellulose conversion to value-added products. This approach will develop high-throughput systems and synthetic biology approaches to realize stable synthetic consortia that route lignocellulosic carbon into short- and

medium-chain fatty acids (SCFAs/MCFAs) rather than methane. Key research objectives are to: (1) design and predict anaerobic fungal and bacterial consortia that efficiently convert lignocellulosic biomass into MCFAs; (2) understand how fermentation parameters and microbe–microbe interactions regulate and drive microbiome metabolic fluxes; and (3) use genomic editing to alter the fermentation byproducts of anaerobic fungi and bolster MCFA titers and yields.

Lignocellulose deconstruction and conversion in nature is driven by mixed microbial partnerships. For example, microbes are particularly well-optimized to recycle organic matter in anaerobic habitats, ranging from landfills to intestinal tracts, via interspecies hydrogen transfer and methane release. Compared to aerobic processes, anaerobic digestion can far more efficiently convert substrate to chemical products. This is largely because much less carbon is funneled to cell growth, resulting in higher yields, and far fewer energy inputs are required because pretreatment, aeration, mixing, and heat removal are greatly reduced. Compartmentalizing difficult biomass deconstruction and production steps among specialist anaerobes is an exciting new route to converting biomass into value-added products, especially if consortia can be built predictively and engineered for stability.

Previously, the research team established model bacterial consortia, enriched from the rumen, which convert lignocellulose into high titers of butyrate, a four-carbon (C4) volatile fatty acid (VFA). Metagenomic and metatranscriptomic analyses identified key chain-elongating bacteria in these consortia that maintain high expression of the reverse β-oxidation pathway responsible for production of C4 through C8 VFAs. In parallel, the team demonstrated that anaerobic rumen fungi within the *Neocallimastix* genus are superior biomass degraders that produce optimal substrates for chain elongators including lactate, acetate, and ethanol. Accordingly, partnering anaerobic fungi and chain-elongating bacteria in synthetic consortia represents a novel strategy for maximizing lignocellulose conversion to C4 through C8 VFAs.

Multiple chain-elongating bacteria were screened, and candidates identified that produce VFAs and grow robustly in culture with known fungal metabolites. The research team paired *Pseudoramibacter alactolyticus*, a top MCFA producer, with the anaerobic fungus *Neocallimastix* sp., observing lactate depletion and butyrate and hexanoate production. These strains were paired for several passages and produced consistent metabolic output each time, thus indicating a stable consortium.

Current work involves semi-quantitatively evaluating abundances of consortia members via quantitative polymerase chain reaction (qPCR). The team will also employ RNA sequencing to evaluate differences in gene expression when anaerobic fungi and chain elongators are grown together compared to monoculture, and under different conditions that might increase MCFA production or shift products to longer MCFAs (e.g., such as adding formate into fungal cultures to increase lactate production). These synthetic communities have potential to stably drive conversion of lignocellulose to value-added products.

Anaerobic fungi depend on hydrogenosomes to generate ATP and hydrogen. However, enzymes involved in carbon metabolism and redox balance in hydrogenosomes are not well understood. This accounts for a primary source of uncertainty in genome-scale metabolic models of anaerobic fungi. To address this, the research team isolated hydrogenosomes from *Caecomyces churrovis* via OptiPrep density gradient centrifugation and confirmed expression of an enzyme complex (i.e., NuoEF and HydA) involved in hydrogen production and redox balance in hydrogenosomes, as well as enzymes for pyruvate metabolism (i.e., PFL and PFOR) using NanoPOTS proteomic analysis and enzyme assays. The function of the heterologous generated NuoEF-HydA complex will be explored with enzyme assays to reveal the role of hydrogenosomal PFL and PFOR by inhibiting PFL with a specific synthesized PFL inhibitor. This approach will enhance understanding of anaerobic fungal metabolism and provide essential data for refining the metabolic modeling of consortia.

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Developing Anaerobic Fungal Tools for Efficient Upgrading of Lignocellulosic Feedstocks

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Project Goals: This project develops genetic and epigenetic tools for emerging model anaerobic fungi to identify the genomic determinants of their powerful biomass-degrading capabilities, facilitate their study, and enable direct fungal conversion of untreated lignocellulose to bioproducts.

Anaerobic fungi (*Neocallimastigomycota*) from the digestive tracts of large herbivores are emerging model species for the efficient deconstruction of untreated renewable plant biomass due to their integration of hydrolytic strategies from the bacterial and fungal kingdoms (Solomon et al. 2016). Anaerobic fungi secrete the largest known diversity of lignocellulolytic carbohydrate active enzymes (CAZymes) in the

fungal kingdom, which unaided can degrade up to 60% of the ingested plant material within the animal digestive tract (Seppälä et al. 2017; Youssef et al. 2013). Unlike many other fungal systems, these CAZymes are tightly regulated and assembled in fungal cellulosomes to synergistically degrade plant material, including untreated agricultural residues, bioenergy crops, and woody biomass, with comparable efficiency regardless of composition (Haitjema et al. 2017; Hooker et al. 2018; Solomon et al. 2016; Solomon et al. 2018). Past efforts have characterized remarkable resistance to lignin composition (Hooker et al. 2018), revealed more efficient enzymes for biosynthesis (Hillman et al. 2021a), and created bioprocesses for efficient conversion of agricultural residues to high-value products (Hillman et al. 2021b). More recently, researchers have created the first tools for transient and compartmentalized heterologous gene expression in these species (Hooker et al. 2023).

Engineering efforts exploit the natural competency of anaerobic fungi to uptake exogenous nucleic acids. While daily dosing with exogenous DNA enables transient transformation, this requires significant DNA input. To evaluate whether genomic integration is possible, the team created linear codon-optimized *hph* cassettes that confer hygromycin resistance. A single dose and selection on hygromycin produced several resistant colonies, of which 30% had a stable resistance phenotype. Subsequent molecular characterization confirmed integration via nonhomologous end joining in various loci, including LTR retrotransposons serving as the first example of such in this family.

Researcheres have also sought to identify an autonomous replicating sequence (ARS) for anaerobic fungal plasmid replication and develop self-replicating plasmids. The team is pursuing a number of strategies, including screening shotgun genomic libraries and plasmids from other species for broad host activity. One plasmid that has shown promise is the yeast 2 μm plasmid, a relatively small multi-copy selfish DNA element that resides in the yeast nucleus at a copy number of 40 to 60. Combining AGF-optimized expression cassettes producing eGFP and hygromycin resistance markers, researchers were able to see a steady level of eGFP expression above background levels even approximately 12 days after a single plasmid dose. However, researchers are still unable to maintain a selectable phenotype for long periods with this, implying that the steady-state copy number and/or promoter expression is too low. To address this, researchers are currently expanding the promoter library and evaluating the impact of plasmid features (e.g., GC content, size, etc.) on stability.

Leveraging natural competency to introduce exogenous DNA, researchers have achieved the first simple methods for targeted heterologous expression in anaerobic fungi and are optimizing approaches for stable phenotypes. This growing toolbox for anaerobic fungi forms foundational tools to generate a deeper systems-level understanding of anaerobic fungal physiology while establishing fundamental knowledge about regulation of gut fungal CAZymes. Ultimately, this research enables predictive biology in anaerobic fungi and derives insight into microbial plant deconstruction to advance the development of economical biofuels and bioproducts.

Haitjema, C. H., et al. 2017. "A Parts List for Fungal Cellulosomes Revealed by Comparative Genomics," *Nature Microbiology* **2**, 17087.

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Hillman, E. T., et al. 2021b. "Hydrolysis of Lignocellulose by Anaerobic Fungi Produces Free Sugars and Organic Acids for Two-Stage Fine Chemical Production with *Kluyveromyces marxianus*," *Biotechnology Progress* **37**, e3172.

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Funding Statement: This project is supported by the BER program through the DOE Office of Science under award number DE-SC0022206. A portion of this research was performed under the Facilities Integrating Collaborations for User Science (FICUS) initiative and used resources at the DOE Joint Genome Institute (JGI) and the Environmental Molecular Sciences Laboratory (EMSL), which are DOE Office of Science user facilities. Both facilities are sponsored by the BER program and operated under contract numbers DE-AC02-05CH11231 (JGI) and DE-AC05-76RL01830 (EMSL).

Transgenic Perturbation of Winter-Biased Genes in *Populus*

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Project Goals: To unravel the molecular mechanisms underlying winter maintenance in temperate deciduous tree species and their impacts on woody biomass productivity, thereby advancing bioenergy crop improvement.

Dormant seasons constitute up to half the lifespan of woody perennial crops in temperate climates. While numerous studies have explored the seasonality of vegetative and floral buds, research investigating wood growth in these trees is primarily conducted in the greenhouse or during the summer season. Understanding maintenance and protection of wood-forming tissues during dormant seasons is crucial for improving stress resilience in the face of climate change. Researchers conducted seasonal transcriptome profiling of xylem tissues from mature *Populus deltoides* and young *P. tremula* x *P. alba* INRA 717-1B4 (717) trees. Self-organizing map (SOM) clustering analysis identified gene clusters that display season-specific expression patterns. Summer-based genes showed Gene Ontology enrichment associated with cell wall biogenesis, as would be expected, whereas genes upregulated during the dormant season are associated with vital mechanisms for winter survival. These mechanisms encompass diverse aspects, including cold tolerance, cryoprotectant synthesis, cell membrane stability and integrity, metabolic adjustments, stress response, cell wall modification, growth regulation, various transport processes, and chromatin remodeling.

CRISPR-Cas9 is used to target winter-biased genes for knockout in poplar 717 in order to characterize their functions. Candidate genes selected to date are mainly involved in the metabolism and transport of carbohydrates, which play a key role in antifreeze, dehydration protection, and spring regrowth. Other candidates are involved in nutrient transport and regulation, and protein modification and turnover. Knockout mutants will be monitored for seasonal phenology during field trials. A subset of knockout lines will be subject to metabolomic and transcriptomic profiling. Network analysis will be used to investigate disrupted pathways and ultimately the molecular processes underlying winter maintenance and protection.

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Engineering Novel Microbes for Upcycling Waste Plastic

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Project Goals: The goal is to develop a consolidated biological process to upcycle waste polyethylene terephthalate (PET).

With its versatile applications in many industrial sectors, the global annual production of PET exceeds 70 million tons. However, less than 20% of that volume can be currently recycled. The accumulation of waste PET in the biosphere has become a global pollution concern, motivating the urgent development of technologies to valorize post-consumer PET and tackle the challenge of its end-of-life management. One solution to address this issue is to upcycle waste PET rather than recycle it to generate the same PET typically with low quality. PET upcycling can be achieved by depolymerizing PET into terephthalic acid (TPA) and ethylene glycol (EG) and biologically converting these monomers into value-added products. Owing to its metabolic capacity of degrading EG and TPA, *Rhodococcus jostii* strain PET (RPET) has been developed as a microbial chassis for PET upcycling (Diao et al 2023). However, the scarcity of synthetic biology tools specifically designed for RPET limits the development of the corresponding microbial cell factories for expanding the repertoire of bioproducts from post-consumer PET. To overcome this limitation, researchers have described the development of potent genetic tools in RPET, including: (1) two inducible and titratable expression systems for tunable gene expression; and (2) Serine Integrase-based Recombinational Tools (SIRT) for genome editing. Using these tools, researchers systematically engineer strain RPET to establish

microbial supply chains for the goal of producing chemicals, including lycopene and lipids from post-consumer PET. With these genetic toolkits at hand, subsequently, researchers enhanced lycopene production by introducing the biosynthetic mevalonate pathway. Moreover, this work validated the proof-of-concept of efficiently converting waste PET into biofuels by assembling a functional module to decouple the lipid biosynthesis from nitrogen starvation in RPET (Diao et al. In preparation). Finally, researchers further estimated the potential of PET bio-upcycling by developing a continuous fed-batch fermentation strategy for the coproduction of lycopene and lipids from post-consumer PET. Overall, this work significantly expands the capacity to manipulate and engineer the strain RPET, paving the way for establishing the microbial supply chain that produces chemicals from post-consumer PET.

Diao, J., et al. 2023. "Upcycling of Poly(Ethylene Terephthalate) to Produce High-Value Bioproducts," *Cell Reports* **42**, 111908.

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Model-Guided Design of Synthetic Microbial Consortia for Next-Generation Biofuel Production

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Project Goals: This project aims to establish the foundation for bioproduction using multifaceted microbial communities. Researchers will build metabolic community models of increasing complexity by integrating multiomics datasets. These models will guide engineering designs for optimized production of biofuels from lignocellulosic biomass. Furthermore, the team will use innovative approaches to augment existing communities for improved bioproduction and complete conversion of different biomass feedstocks. Overall, these strategies will provide knowledge of the functional metabolic exchanges driving interspecies interactions in microbial communities, thus providing insights into fundamental biological processes. Lessons learned here will be crucial for researchers' ability to design stable microbial communities for various biotechnology applications in the future.

The multiplicity of intertwined, interspecies interactions within microbial communities regulates their functional organization and assembly. This allows these communities to perform complex functional tasks unreachable by axenic cultures, such as the breakdown of recalcitrant lignocellulosic materials into high-energy volatile fatty acids. Bioproduction of one such fatty acid, butyric acid (BA), from sustainable lignocellulosic sources has gained attention owing to BA's versatile applications as a precursor for a range of products, including sustainable aviation fuel, polymers, fibers, and cosmetics. However, the current necessity for costly enzymatic pretreatment of the lignocellulosic substrate that is currently required undermines the economic advantages of a biosustainable process.

A mutualistic coculture of the thermophilic strains *Clostridium thermocellum* and *C. thermobutyricum* was recently shown to be effective in converting lignocellulose to BA without expensive enzymatic pretreatment (Chi et al. 2018). However, the process is still suboptimal, leaving ample room for improvement in substrate utilization and product formation. While the coculture of *C. thermocellum* and *C. thermobutyricum* resulted in a >100% improvement in substrate utilization compared to a monoculture of *C. thermocellum*, notable amounts of carbohydrates—primarily consisting of xylans—and the fermentation end products ethanol and acetate, were left unutilized.

Here, researchers characterized the metabolic interactions and exchanges of this thermophilic coculture using high-quality, manually curated genome-scale metabolic models for both species. Compartmentalized as a community metabolic model (CM-model) comprising 1,777 reactions, 1,679 metabolites, and 1,569 genes, the constructed CM-model enabled researchers to identify predicted metabolic bottlenecks that account for the coculture's constrained BA production and incomplete substrate utilization. The group aimed to release these bottlenecks via targeted and untargeted augmentation of the community to generate a reproducible synthetic community (SynCom), thereby improving substrate utilization and BA production. Targeted augmentation involves conducting bibliographic research and using CM-model guidance to select characterized microbial strains compatible with the system. Conversely, untargeted expansion entails isolating and selecting microbes with desirable metabolic traits.

For the targeted augmentation, the researchers considered specific roles fulfilled by individual members of the coculture (with *C. thermocellum* specializing in recalcitrant carbohydrate biomass hydrolysis and *C. thermobutyricum*

in BA production from soluble sugars) and structured the expanded SynCom around functional modules. This organizational framework integrates *Thermoanaerobacterium xylanolyticum* along with *C. thermocellum* into a "lytic" module, specializing in complex carbohydrate oligomer breakdown. *Moorella thermoacetica* was chosen for the "scavenging" module, aimed at recapturing and redirecting residual byproducts. Together, these augmented modules combined with the "yield" module, focused on *C. thermobutyricum*-mediated BA production, completed the setup.

Simultaneously, researchers explored augmenting the community in an untargeted approach. Soil samples were collected and grown on lignocellulosic substrates of varying recalcitrance under thermophilic, anoxic conditions to enrich thermophilic bacteria capable of hydrolyzing the complex oligomers in lignocellulose. Additional selection pressures were applied by growing these enrichments on spent supernatants from the coculture's growth on deacetylated and mechanically refined corn-stover (DMR). From these, the group isolated and characterized thermophilic strains capable of growing solely on DMR and other non-pretreated lignocellulosic substrates, indicating possible new metabolic capabilities. Next, the group constructed three-member communities from these strains by pairing them with *C. thermocellum* and *C. thermobutyricum* and observed improved BA titers from DMR compared to the coculture. Hence, this study establishes the foundation for advanced bioproduction using multifaceted microbial communities.

Chi, X., et al. 2018. "Hyper-Production of Butyric Acid from Delignified Rice Straw by a Novel Consolidated Bioprocess," *Bioresource Technology* **254**, 115–20. DOI:10.1016/j.biortech.2018.01.042.

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A Gene-Editing System for Large-Scale Fungal Phenotyping in a Model Wood Decomposer

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Project Goals: This project combines CRISPR-Cas9-based genome-editing and network analysis for large-scale phenotyping in a model wood decomposer fungus relevant to the DOE mission area. The overall goal is to develop a high-throughput genetic platform that enables discovery of distinctive genes and genetic features that speed wood degradation by brown rot fungal species. The research endeavors to provide stand-alone tools and resources for discovering novel fungal genetic mechanisms that can be used together to advance relevant plant biomass conversion research in the post-genomic era.

This research focuses on a group of unique wood decomposer basidiomycete fungi—brown rot fungi—that harbors industrially relevant pathways for extracting carbohydrates from lignocellulose and have broad relevance to global carbon cycling. Distinct from other fungi, brown rot species use nonenzymatic reactive oxygen species mechanisms to modify lignin and selectively extract sugars. Their degradative mechanisms, from a process efficiency standpoint, represent a pathway upgrade relative to the ancestral approaches in white rot species (Hibbett and Donoghue 2001; Eastwood et al. 2011). Fungi obtained this capacity evolutionarily by shedding rather than gaining carbohydrate-active enzymes repertoire genes (Martinez et al. 2009; Floudas et al. 2012; Riley et al. 2014). This paradox therefore makes brown rot fungi a promising candidate for discovering unknown genetic mechanisms governing plant biomass degradation. Although DOE mission relevance is clear and major genomically informed advances in brown rot have been achieved, progress is limited by an inability to manipulate genes in any brown rot fungal strain.

The potential key roles of fungal genome reshuffling and gene regulation in determining brown rot efficacy are widely recognized (Zhang et al. 2016; Zhang et al. 2017; Zhang et al. 2019). Using functional genomic tools, a staggered two-step (i.e., oxidation-then-hydrolysis) gene regulation model for brown rot was elucidated in the *Proceedings of the National Academy of Sciences* (Zhang et al. 2016) and *mBio* (Zhang et al. 2019). Although these genomic studies have greatly advanced understanding of brown rot, its genetic basis

remains uncharacterized and unharnessed. For example, (1) gene function in the two-step model remains unverified and ambiguous, (2) the gene regulatory mechanism used to control and consolidate the two steps is unclear, and (3) the functions of most genes identified by multiomics are either hypothetical or unknown. The existence of these gaps is primarily due to the lack of a robust genome-editing tools for validating and discovering brown rot genetic features.

This project will integrate systems biology, genome editing, and network modeling to address these key gaps. Three project objectives include:

Objective 1: Create a CRISPR-Cas9-mediated gene-editing system and use it to target genes. To genetically manipulate brown rot fungal species, the research team first created a DNA transformation procedure in a model species—*Gloeophyllum trabeum*. A series of genetic tools were then developed to test and control gene expression in the fungus, including a collective of promoters, a laccase reporter system for reporting extracellular protein function (Li et al. 2023), and a green fluorescent protein reporter system for testing intracellular protein function and nuclear localization signals and for localizing the cellular loci of lignocellulolytic enzymes. The strain's dikaryotic genome was resolved using long-read PacBio sequencing to enable gene editing on both alleles. A preassembled Cas9-single guide RNA (sgRNA) ribonucleoprotein method was attempted to target benzoquinone reductase, a key Fenton gene. Mutants with successful disruptions of one or two alleles were obtained. Mutation mechanisms involved in the editing process were studied. Although editing efficacy is low (2% to 5%), the method is acceptable to test brown rot gene functions at a singular gene level (e.g., by targeting crucial candidate genes pinpointed by omics and network analysis).

Objective 2: Model a carbon-utilizing network governing brown rot and use it to mine decay genes. To build a carbon-utilizing gene network for discovering novel brown rot genetic features, transcriptome response to a broad spectrum of lignocellulose derivative carbon sources was measured in two brown rot species, *G. trabeum* and *Rhodonia placenta*. This species comparison enabled identification of shared or distinct mechanisms. Different network-analyzing tools were tested and compared, and key modules and their "hub" genes associated with lignocellulose polymers or monomers were identified. DNA affinity purification (DAP-seq) was then used to identify the cis- and trans-regulatory elements involved in the carbon signaling pathway, and the key regulatory machinery unique to brown rot was revealed (Zhang et al. 2022). Networks derived from gene co-expression and DAP-seq were overlapped. In the context of the full project, this objective will complement the gene targets for large-scale phenotypic screening.

Objective 3: Develop multiplexed genome editing for large-scale phenotypic screens. This objective aims to develop a pipeline to use the multiplexing sgRNA library for genome editing and mutant library construction for large-scale phenotypic screens, followed by next-generation sequencing to discover key functional genes. An all-in-one Cas9 and sgRNA expression construct was built and used to target genes. Several candidate genes were selected for disruption experiments to test the method's editing efficiency. Insertion frequency of the gene constructs was studied. Moving forward, the multiplexed sgRNA library will be expressed in *G. trabeum* to specifically study the pathways associated with lignin utilization revealed by network analysis as a step toward large-scale phenotypic screening.

This project aims to provide stand-alone tools and resources to elucidate fundamental microbial processes relevant to the DOE mission area, advancing new engineering designs for lignocellulose bioconversion.

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Science Focus Area
IMAGINE BioSecurity: Integrative Modeling and Genome-Scale Engineering for Biosystems Security

Mesocosm-Based Methods to Evaluate Biocontainment Strategies and Impact of Industrial Microbes Upon Native Ecosystems

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https://genomicscience.energy.gov/nrel-imagine/

Project Goals: The Integrative Modeling and Genome-Scale Engineering for Biosystems Security (IMAGINE BioSecurity) Science Focus Area seeks to establish an understanding of the behavior of engineered microbes in controlled versus environmental conditions to predictively devise new strategies for responding to biological escape. To this end, the IMAGINE team has established a plant–soil mesocosm platform to track and quantify the fate of industrial microbes in environmental systems and assess the efficacy of biocontainment constraints upon genetically engineered microbe escape frequency and the impact of industrial microbes upon native ecological microbiomes.

Genetically modified industrial production microbes and their associated bioproducts have emerged as an integral component of a sustainable bioeconomy. However, the rapid development of these innovative technologies raises biosecurity concerns, namely, the risk of environmental escape. Thus, the realization of a bioeconomy hinges not only on the development and deployment of microbial production hosts, but also on the development of secure biosystems and biocontainment designs. Current laboratory-based biocontainment testing systems do not accurately reflect complexities found in natural environments, necessitating an environmentally relevant analysis pipeline that allows for the detection of rare escapees, the effects of associated bioproducts, and the impacts on native ecologies. To this end, the team has developed an approach that utilizes soil mesocosms and integrated systems analyses to evaluate the efficacy of novel biocontainment strategies and assess the impact of production systems upon terrestrial microbiome dynamics. The project demonstrates the utility of this approach by modeling a contamination with industrial microbial chassis versus their biocontained counterparts. Here, researchers demonstrate the broad utility of this system by highlighting findings from both strains of *Saccharomyces cerevisiae* that are contained with an inducible toxin–antitoxin system and strains of *Escherichia coli* that are contained via genomic recoding. The resultant data demonstrate that this system has broad utility across diverse microbial chassis and biocontainment strategies. The data also enable tracking the fate of the contaminating microbe with high sensitivity in the soil and monitoring broader impacts of the perturbation on the underlying soil system. The findings presented here support the use of this mesocosm-based approach to assess the environmental impact of industrial microbes and to validate biocontainment strategies.

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IMAGINE BioSecurity: Genome-Scale Engineering and High-Throughput Screening to Establish Secure Biosystems Design

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https://genomicscience.energy.gov/nrel-imagine/

Project Goals: The Integrative Modeling and Genome-Scale Engineering for Biosystems Security (IMAGINE BioSecurity) Science Focus Area project seeks to establish a predictive framework for secure biosystems design. To this end, the IMAGINE team integrates core capabilities in synthetic and applied systems biology to develop a high-throughput platform for the design, generation, and analysis of biocontainment strategies in industrially relevant and emerging next-generation microbes.

Microbial biocatalysts and bioderived products have emerged as an integral component of a sustainable bioeconomy, with an array of applications in agriculture and bioenergy. However, the rapid development of genetically engineered microbes and associated synthetic biology approaches raises several biosecurity concerns related to microbial environmental escape, detection, and impact upon native ecosystems (Arnolds et al. 2021; Arnolds et al. 2024). To establish a secure bioeconomy, novel biocontainment strategies that do not compromise laboratory performance are needed. To this end, the IMAGINE BioSecurity team was established to accomplish the overarching goal of achieving predictive control of engineered systems to enable secure biosystems design. The team is developing an array of passive and active synthetic biocontainment strategies in a series of nonmodel, industrial, and next-generation microbial hosts to serve as chassis for secure biosystems design.

To facilitate the analysis of combinatorial constructs in the target organisms, a method termed combinatorial genetics en masse (CombiGEM; Wong et al. 2016; Hernandez Hernandez et al. 2023), for generating combinatorial genotypes en masse and tracking them in mixed populations using DNA barcodes and next-generation sequencing, was implemented. Combinatorial biocontainment strategies are being developed and evaluated for the capacity to reduce genetically modified organism (GMO) escape frequency in laboratory and environmental simulation settings. Additional efforts to target synthetic carbon, nitrogen, and phosphorus storage auxotrophies are under development (Sebesta et al. 2022; Sebesta et al. 2024). In parallel, researchers have initiated assessment of the metabolic burden associated with implementation of these strategies, with the goal of maximizing biocontainment while maintaining optimal microbial fitness in deployment settings. Engineered strains are experimentally analyzed via growth, escape frequency, and bioproductivity using high-throughput screening in laboratory and environmental mesocosm settings. Strains are concurrently subjected to fitness and escape frequency screening assays to assess the effects of genetic safeguards on strain fitness and biocontainment efficacy.

Systems-level analyses of these microbial biocatalysts in the absence and presence of biocontainment constraints will elucidate principles that (1) govern effective biocontainment and laboratory performance and (2) drive biological systems in their natural environments. These learnings will establish an extensive library of biocontainment modules and strains, a testing platform, and systems knowledgebase, and lay the foundation for predictive design of biocontainment strategies with enhanced stability and resilience in diverse microbial hosts. Combined, these efforts will reduce the risks associated with deployment of GMOs, ultimately accelerating a secure bioeconomy.

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Computational Modeling to Enable Predictive Secure Biosystems Designs

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Project Goals: Systems modeling is an integral part of the Integrative Modeling and Genome-Scale Engineering for Biosystems Security (IMAGINE BioSecurity) Science Focus Area project that addresses biosafety concerns related to microbial biocontainment and performance stability. Researchers aim to develop integrative bioinformatics tools for the efficient modeling of metabolism and gene expression (ME-models) of bacterial systems and metabolic flux analysis (MFA). These ME-models and MFA analyses will be used for the predictive design of novel containment strategies by identifying critical metabolic reactions governing secure biosystems designs in engineered bacteria.

Genetically modified microorganisms (GMMs) are widely used in agriculture and bioenergy industries. Among current systems biology tools, computational methods can interrogate systems with unprecedented detail and high throughput. However, lagging behind is the application of these tools for secure biosystems design. Here, the group presents coralME, FreeFlux, and EMUlator2ML, new computational tools aiming to design predictable and generalizable biocontainment and robustness stabilization strategies.

First, the team developed coralME to automatically reconstruct nearly finished ME-models from genome-scale metabolic models (M-models), which allowed researchers to complete four highly curated ME-models for bioeconomy-relevant microorganisms *Pseudomonas putida* KT2440 (*i*Ppu1686-ME), *Synechocystis* sp. PCC 6803 (*i*Syn1015-ME), *Clostridium ljungdahlii* DSM 13528 (*i*Clj978-ME), and *Mycoplasma mycoides* JCVI-Syn3A (*i*Mmy259-ME). As they include gene expression, the number of components and reactions grows to accommodate transcription and translation. For instance, *i*Ppu1686-ME models 224 additional gene products (+15.32%) compared to its parental M-model, *i*JN1463, in a network of 14,426 reactions and 7,566 components, increasing by 392.86% and 251.42% the number of reactions and components, respectively. Additionally, coralME aided the development of 17 draft ME-models for diverse bacteria, covering eight phyla. Application of coralME to these 21 different organisms resulted in short reconstruction times, effectively reducing the reconstruction of ME-models from several months to minutes. The platform is ideally suited for reconstruction of feasible ME-models employing efficient troubleshooting and reporting methods that repair and guide the manual addition of reactions. Consequently, coralME has accelerated modeling and simulation, further allowing the prediction of hundreds of essential genes, microbe–microbe interactions, overflow metabolites, use and essentiality of enzyme cofactors, and proteome composition.

In parallel, the team have developed FreeFlux, an open-source Python package which offers comprehensive ^{13}C-MFA analysis, boasting swift, reliable flux computation. EMUlator2ML, a machine learning framework, accelerates flux estimation, enhancing large-scale analysis and strain screening by "learning" intrinsic relationships between metabolite labeling patterns and metabolic flux, inferring fluxomic phenotypes from isotopically metabolomic datasets. It demonstrated the ability to use M-models to generate the training dataset with minimal reliance on experimental data. Finally, the group is introducing a biocontainment approach combining ensemble computational modeling with CRISPR interference (CRISPRi) to modulate GMM metabolism, targeting core robustness for growth instability. This approach enables identification of enzymatic targets sensitive to expression perturbations and establishing genetic circuits for enhanced performance and safety, with strains meeting the National Institutes of Health escape frequency standard, validated across various conditions.

In summary, researchers used ensemble modeling, FreeFlux, EMUlator2ML, and highly curated M-models and ME-models to elucidate microbial metabolism under variable conditions, metabolic interactions within microbiomes, and the productivity of GMMs under secure biosystems constraints in an iterative design-build-test-learn cycle. The resultant pipeline will enable rapid and predictive secure biosystems designs.

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Science Focus Area
Microbial Secure Biosystems Design

Massive Protein Redesign to Make Overlapping Genes

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The future bioeconomy requires engineered microbes that behave predictably, robustly, and safely in natural environments. Most engineered microbes, however, fail to function outside of the laboratory and carry uncontrolled risks of genetic pollution into natural gene pools. The BioSecure Science Focus Area at Lawrence Livermore National Laboratory is developing new approaches to enhance biocontainment of engineered bacteria.

Introducing overlapping genes into engineered bacteria can enhance stability and productivity, while reducing the risk of uncontrolled genetic spread. Overlapping genes share a single coding sequence of DNA and RNA but are translated in alternate reading frames. By designing overlapping genes, researchers align their evolutionary trajectories towards desirable traits. For example, overlapping an essential gene can prolong an engineered function and overlapping a toxic gene can reduce horizontal gene transfer. However, creating functional overlapping sequences requires extensive protein redesign and remains technically and computationally challenging.

The team performed a large-scale computational screen between 118 genes by generating over 7 million overlapping sequences *in silico*. Researchers compared these overlapping sequences and their predicted function (scores) between gene pairs to identify genes and gene pairs more amenable to overlap. Genes vary in their malleability, and the resulting scores are influenced by several factors including gene length, number of orthologs, and amino acid content. Several small genes, such as *hicA*, *infA*, and *purE*, appear amenable to overlap with multiple partner genes. Several larger genes, such as *lacZ* and *aroB,* also score well when overlapped with multiple smaller partners. These results suggest designed overlaps are feasible for many genes. However, these predictions are based on unproven computational models of protein function derived from evolutionary sequence data and must be experimentally validated.

To validate the protein models, researchers have begun experimentally testing redesigned protein variants for individual proteins identified in the screen. Given that most genes in the screen are conditionally essential in *E. coli,* the team tested protein function by complementing growth in auxotroph strains that lack these genes. The team has implemented a pipeline to test pooled variants at scale by sequencing and then use these fitness data to interpret model predictions.

In small-scale trials, researchers have identified functional genes that have undergone significant redesign but retain function. For example, *purE* variants exhibited wildtype function despite over 40% of residues being altered, while *hisI* variants maintained wildtype function with ~50% residues changed. In a recent trial, all tested *hicA* sequences (6/6) were functional, with each variant featuring 30% to 50% changed residues. Overall, researchers have experimentally validated that the protein models for multiple genes can

create sequence-diverse yet functional variants, with successful validation observed in 10 out of the14 genes tested.

In the next phase, researchers will expand the experimental throughput by testing thousands of variants for select genes while also testing the function of overlapping sequences for both genes. These results demonstrate the feasibility of computational redesign of entire proteins in support of designed gene overlap. This ability to create novel overlapping genes will foster the next generation of dependable and secure engineered microbes.

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Principles Governing Expression of Overlapping Genes

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Project Goals: A primary goal of the Lawrence Livermore National Laboratory BioSecure Science Focus Area is to establish gene overlaps—in which two genes are encoded within the same DNA sequence through use of alternative reading frames—as a generalizable biocontainment strategy to protect engineered functions against mutational inactivation and to mitigate the horizontal transfer of invasive genes. This project is focused on determining the biophysical and molecular principles governing expression of overlapping genes. The project seeks to (1) identify the biophysical mechanisms and constraints underlying expression of overlapping genes and (2) predict and engineer future overlapping genes used in microbes for deployment.

In synthetic biology, methods for stabilizing genetically engineered functions in intended hosts are necessary to cope with mutation accumulation. One generalizable strategy to preserve genetic information is through gene overlaps, translating two distinct proteins from the same mRNA in different open reading frames. Overlapping a sequence with an essential gene can alter its fitness landscape and produce a constrained evolutionary path. While ongoing work is focused on large-scale redesign of the entangled proteins to satisfy sequence constraints required by sequence overlap, little is known about how expression is affected for entangled genes and its ramifications on the success of developing successful gene entanglements. To dissect the role of entanglement on gene expression, researchers have devised a methodological pipeline of genetic entanglement by inserting a protein encoded in an alternate reading frame with an external gene, thereby minimizing amino acid changes to both genes and permitting functionality of both overlapped genes. Researchers demonstrate the creation and evaluation of multiple overlapping, out-of-frame insertion designs in flexible loops of inner-membrane anchored antibiotic-resistance alleles encoding efflux pumps. The team shows that inserted genes (toxins and fluorescent reporters) can function despite their location inside another coding sequence in an alternate frame and that function of both genes can be retained with minimal redesign. Interestingly, the team finds that the nested genes exhibit significant variability in the expression based on the location of the insertion of the external gene. This variability is not due to differences in mRNA levels but manifests at the level of protein abundance. To further generate a broad understanding of expression alterations during gene-nesting, researchers have performed insertional profiling to generate libraries of additional external genes (encoding for cytoplasmic globular proteins) with a nested green fluorescent protein (*gfp)* gene located throughout the sequence of the external gene. After identifying nested entanglements with full functionality of the external gene, the team will perform a series of mechanistic studies (Structure-Seq and Ribo-Seq) to identify whether and how expression may be altered for both entangled genes. These studies will provide general principles that underlie the expression of engineered entangled and nested genes with the goal of creating entangled genes capable of expressing at levels needed to stabilize function of both genes. Ultimately, this work will establish general guidelines for designing gene entanglements for improved stability of engineered genetics and circuits in microbes deployed *in situ*.

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Mapping Toxin-Antitoxin Systems for Microbial Community Biocontainment

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Project Goals: The Lawrence Livermore National Laboratory Secure Biosystems Design Science Focus Area aims to develop robust biosecurity tools at the sequence, cellular, and population levels to safeguard the deployment of genetically engineered bacteria for environmental applications. This project aims to exploit the competition between toxin–antitoxin (TA) systems to model and ultimately regulate horizontal gene transfer (HGT) within microbial communities for biocontainment.

Bacteria rapidly disseminate genetic information through HGT, a fundamental driver of microbial evolution. While there is enormous potential in the development of engineered microbial products that are compatible with native microbiota in target environments (e.g., gut or rhizosphere microbiomes), the challenge lies in controlling HGT to and from deployable engineered bacteria. This raises important questions regarding the maintenance of genetic stability over time, and the ecological containment of genetically modified organisms as well as the recombinant or synthetic constructs they harbor. Rather than attempting to suppress natural HGT *in situ*, the team's goal is to examine the forces and barriers that shape HGT networks in microbial populations, and to leverage the principles uncovered to develop genetic tools that promote genetic stability of deployable engineered microbes.

The ubiquitous and mobile nature of TA systems in prokaryotes makes them versatile effectors of biocontainment mediated through HGT network interactions. Researchers systematically identified and mapped 40,000 TA systems onto the global bacterial plasmidome, discovering how TA systems are organized through HGT communities, rather than traditional taxonomic classifications. Machine learning models trained on the most common 10% of TA systems alone were able to assign plasmids to HGT communities with 95% accuracy, suggesting each HGT community has its own unique and predictable TA signature. The results of this study imply that HGT networks are constrained, at least in part, by the compatibility between TA systems and provide a coherent explanation for the otherwise erratic distribution across microbial genomes. Understanding and leveraging the dynamics of this innate competition between TA systems could form the basis of a TA-based mechanism for custom community-level invasion or biocontainment. Initial models will inform experiments to test the potential and limitations of TA-based design for controlling the horizontal spread of engineered plasmids outward and natural plasmids inward, in both simple and complex microbial consortia.

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Science Focus Area
Persistence Control of Engineered Functions in Complex Soil Microbiomes

Drought-Induced Plant Physiology Drives Altered Microbe–Metabolite Interactions Along the Plant Rhizosphere Column

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Project Goals: The Persistence Control Science Focus Area (PerCon SFA) at Pacific Northwest National Laboratory (PNNL) seeks to understand plant–microbiome interactions in bioenergy crops to establish plant growth–promoting microbiomes that are contained to the rhizosphere of a target plant. This vision requires the discovery of exudate catabolism pathways from plant roots, the elimination of genes that support fitness in bulk soil environments without decreasing rhizosphere fitness, and the engineering of rhizosphere-niche occupation traits in phylogenetically distant bacteria. The team anticipates the impacts of these efforts will be to increase understanding of plant–microbe interactions and to extend high-throughput systems and synthetic biology tools to nonmodel microbes.

The rhizosphere represents a critical zone of interaction between plant roots and soil microbiota, harboring complex biotic interactions that are essential for plant and soil health. The intricate nature of these interactions becomes particularly evident under environmental stress conditions such as drought. Though scientists know the soil microbiome changes as soil depth increases, previous research identifying drought-induced shifts in microbial abundance and root exudate composition used homogenized rhizosphere samples, losing spatial resolution.

With the increasing prevalence of drought conditions due to climate change, it is imperative to understand its impact on the rhizosphere. This study aims to elucidate the changes in microbe–metabolite interactions along the rhizosphere column of the bioenergy crop sorghum under drought stress.

The RhizoGrid, a spatial root cartography experimental system, was deployed to monitor variations in root physiology, microbial community assembly, and interactions with root exudates using planar and axial spatial sampling under drought and control conditions.

Investigation reveals a significant spatial organization within the healthy rhizosphere. Largely influenced by the enrichment of multiple microbial taxa at shallow depths, *Flavisolibacter*, *Lysobacter*, and *Ramlibacter* genera exhibited noticeable spatial variation, decreasing in abundance in the lower half of the rhizosphere soil column. Comprehensive analysis highlighted drought-induced shifts in rhizosphere community composition, with marked decreases in taxa diversity and root exudate complexity, and an increase in intraplanar beta diversity across depth. Alterations in plant root physiology, characterized by reduced root mass and number along the RhizoGrid soil column, and machine learning network analysis of spatial microbe–metabolite patterns define the assembly of a drought-distinct microbiome state in the lower half of the soil column marked by a depletion of various Proteobacteria and a reduction in classes of benzenoid metabolites.

These findings underscore the importance of spatial resolution in assessing the rhizosphere's response to drought, providing valuable insights into the resilience of soil ecosystems and recontextualizing previous work. The team believes this approach will be a model for high-resolution investigation of plant–microbe interactions subjected to environmental stress or the introduction of beneficial or pathogenic agents.

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Reinforced CRISPR Interference (CRISPRi) Enables Reliable Multiplex Gene Repression in Phylogenetically Distant Bacteria

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Project Goals: The Persistence Control Science Focus Area (PerCon SFA) at PNNL seeks to understand plant–microbiome interactions in bioenergy crops to establish plant-growth-promoting microbiomes that are contained to the rhizosphere of a target plant. This vision requires the discovery of exudate catabolism pathways from plant roots, the elimination of genes that support fitness in bulk soil environments without decreasing rhizosphere fitness, and the engineering of rhizosphere niche occupation traits in phylogenetically distant bacteria. Researchers anticipate the impacts of these efforts will be to increase understanding of plant–microbe interactions and to extend high-throughput systems and synthetic biology tools to nonmodel microbes.

Persistence control is an engineering approach in which survival of genetically modified microorganisms is restricted to a target environmental niche. However, the current knowledge of gene functions is insufficient to rationally design persistence control traits. CRISPR interference (CRISPRi) uses the combination of a Cas protein and a guide RNA to repress gene expression in a sequence-specific manner and can be used to identify genes involved in growth and survival.

However, current CRISPRi tools each have substantial caveats that limit their applications outside of simple, well-controlled laboratory conditions. The PerCon SFA is using the sorghum rhizosphere as a target environmental niche and has developed a multi-guide CRISPRi system to simultaneously repress multiple genes in this complex environment. Researchers use Serine recombinase-Assisted Genome Engineering (SAGE) to simultaneously integrate the CRISPRi protein dCas12a and its guide array into the host chromosome. Fine-tuning the expression of each component is critical to maximize gene repression and minimize fitness defects. Unlike dCas9, the most common CRISPRi system, the primary CRISPR array transcript processed by dCas12a creates distinct guide RNAs, enabling researchers to repress multiple genes with a single transcriptional unit. To address the unpredictable efficiency of single-guide repression, researchers show that reinforcing CRISPRi with multiple guides per gene greatly improves both the magnitude and reliability of gene repression for fluorescence and cell-growth gene targets. SAGE is an organism-agnostic toolkit that enables the creation of robust CRISPRi systems in bacteria from multiple phyla.

The team is currently using CRISPRi in the rhizobacteria *Pseudomonas facilor* and *Pseudomonas fluorescens* to prototype multi-gene deletions and perform high-throughput functional screens. Pooled multiplex CRISPRi screens enable researchers to evaluate whether pairs of genes that conditionally impact fitness are involved in the same or distinct physiological processes under abiotic stresses. Additionally, researchers have begun comparing the outcomes of genome-scale single guide CRISPRi, multiple guide CRISPRi, and randomly barcoded transposon mutant screens. Early results highlight advantages and weaknesses to each approach and have found that co-analysis of data from distinct genome-wide screens can identify proteins with independently functioning protein domains.

Elmore, et al. 2023. "High-Throughput Genetic Engineering of Nonmodel and Undomesticated Bacteria Via Iterative Site-Specific Genome Integration," *Science Advances* **9**(10), eade1285. DOI:10.1126/sciadv.ade1285.

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Bacterial Degradation of Sorgoleone, a Step Towards Enforcing Rhizobacteria Containment

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Project Goals: The Persistence Control Science Focus Area (PerCon SFA) at Pacific Northwest National Laboratory seeks to understand plant–microbiome interactions in bioenergy crops to establish plant growth–promoting microbiomes that are contained to the rhizosphere of a target plant. This vision requires the discovery of exudate catabolism pathways from plant roots, the elimination of genes that support fitness in bulk soil environments without decreasing rhizosphere fitness, and the engineering of rhizosphere niche occupation traits in phylogenetically distant bacteria. Researchers anticipate the impacts of these efforts will be to increase understanding of plant–microbe interactions and to extend high-throughput systems and synthetic biology tools to nonmodel microbes.

Metabolite exchange between plant roots and their associated rhizosphere microbiomes underpins plant growth promotion by microbes. Root tips of the bioenergy crop *Sorghum bicolor* exude large amounts of a lipophilic benzoquinone called sorgoleone. This allelochemical suppresses the growth of competing plant seedlings and is slowly mineralized by microbes in soil. As an avenue to understanding how sorghum and its root microbiome may be connected through root exudates, the group identified the molecular determinants of microbial sorgoleone degradation and the distribution of this trait among microbes. The team isolated and studied three bacterial strains from sorghum-cultivated soils that were classified as *Acinetobacter*, *Burkholderia*, and *Pseudomonas* species able to grow with sorgoleone as a sole carbon and energy source. The genomes of these strains were sequenced and subjected to transcriptomic and gene fitness analyses to identify candidate sorgoleone degradation genes. Follow-up mutational analysis showed that sorgoleone catabolism is dependent on four contiguous genes that are conserved among the strains the teams sequenced. Researchers refer to these four genes as the *srg* (sorgoleone degradation) cluster. Phylogenetic analysis of the *srg* cluster using Snekmer showed that sorgoleone catabolism is enriched in sorghum-associated *Streptomyces* strains over isolates from the *Populus* rhizosphere. The discovery of bacteria that grow on a compound like sorgoleone that is plant specific and not widely distributed in the environment provides an opportunity for the PerCon SFA to study how sorghum exudates can enforce the development of a rhizosphere-specific microbiome for the mutual benefit of plant and microbe.

Chang, C. H., et al. 2023. "Snekmer: a Scalable Pipeline for Protein Sequence Fingerprinting Based on Amino Acid Recoding," *Bioinformatics Advances* **3**(1). DOI:10.1093/bioadv/vbad005.

Oda, Y., et al. 2023. "Sorgoleone Degradation by Sorghum-Associated Bacteria; An Opportunity for Enforcing Plant Growth Promotion," *bioRxiv*. DOI:10.1101/2023.05.26.542311.

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Science Focus Area
Secure Ecosystem Engineering and Design (SEED)

Understanding Microbial Invasion Biology from Laboratory-to-Field for Secure Ecosystem Engineering and Design

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Project Goals: The Secure Ecosystem Engineering and Design (SEED) Science Focus Area (SFA), led by Oak Ridge National Laboratory, combines unique resources and expertise in the biochemistry, genetics, and ecology of plant–microbe interactions with new approaches for analysis and manipulation of complex biological systems. The long-term objective is to develop a foundational understanding of how nonnative microorganisms establish, spread, and impact ecosystems critical to DOE missions. This knowledge will guide biosystems design for ecosystem engineering while providing the baseline understanding needed for risk assessment and decision making.

The deliberate introduction of plants or microbes into new environments will be necessary to address national and global energy and environmental challenges. However, scientists currently lack the knowledge and tools necessary to successfully predict and introduce beneficial alterations, prevent undesired modifications, or predict the risks of proposed ecosystem engineering efforts.

A promising strategy for ecosystem engineering is the deliberate introduction of microbes to produce a specific effect on ecosystem function. At the same time, the anthropogenic-assisted movement of microbes and changes in climate are accelerating the emergence of nonnative pathogens in resident communities. Regardless of the source, biosystems design strategies must accommodate and encompass the dynamic ecological and evolutionary factors that determine the outcome of natural and engineered invasions into ecosystems. Accounting for these barriers and their dynamics will enable new engineering approaches to safely manipulate the introduction of genes, pathways, and microbes into ecosystems to solve critical environmental challenges while limiting undesired community perturbations.

For ecosystem engineering using plant growth–promoting bacteria, the project has identified several nonmodel strains of *Bacillus* as testbeds for secure biodesign and genome engineering. While *Bacillus* species are abundant in soils and plant tissues and are common components of commercially available biological control products, there is a growing concern that the deliberate introduction of microbes into the environment will have unintended consequences on ecosystem health. Additionally, the mechanisms underlying the establishment and impact of introduced microbes for ecosystem engineering on plant and ecosystem health are not well understood. Therefore, the team has assessed the establishment and persistence of several *Bacillus* spp. across a series of laboratory-to-field experiments. For these experiments, researchers have benchmarked the persistence of these nonmodel *Bacillus* spp. in the soil microbiomes of *Populus* and quantified their impacts on the host and resident microbiomes.

Furthermore, there is a growing concern that the anthropogenic movement of plants and their associated microbes will accelerate the emergence of novel pathogens. Microbial functions are notoriously context dependent and, with increasing movement of microbes and changes in climate, organisms are likely to transition from mutualist to pathogen with increased frequency. One such example is the fungal pathogen *Sphaerulina musiva* which following the human translocation of *Populus* across North America, spread from its original host, *P. deltoides,* to novel hosts including *P. balsamifera* and the DOE-flagship species *P. trichocarpa*. In its new hosts, *S. musiva* induces fatal stem cankers in natural and managed settings that can greatly inhibit plant production. As a result, researchers have also assessed the effects of *S. musiva* establishment on host plants and their associated microbial communities in laboratory-to-field experiments.

Given the commercial applications of *Bacillus* spp. as a biofungicide, the team has tested the interactions between nonmodel *Bacillus* strains against several natural isolates of *S. musiva*. This characterization has uncovered a range of inhibitory strengths and varying tolerances for *Bacillus* and *S. musiva*, respectively. Using these results, researchers are

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developing a high-throughput image-based method paired with metabolomics to understand the genetic and chemical diversity for biocontrol.

Collectively, obtaining information on the direct or indirect mechanisms that control microbial-based biocontrol targeting fungal pathogens can help improve biodesign strategies aiming to increase *Populus* productivity and sustainability.

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Synthetic Biology Tools to Reliably Establish and Monitor Microbial Invasions in the Rhizosphere

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Project Goals: The Secure Ecosystem Engineering and Design (SEED) Science Focus Area (SFA), led by Oak Ridge National Laboratory, combines unique resources and expertise in the biochemistry, genetics, and ecology of plant–microbe interactions with new approaches for analysis and manipulation of complex biological systems. The long-term objective is to develop a foundational understanding of how nonnative microorganisms establish, spread, and impact ecosystems critical to DOE missions. This knowledge will guide biosystems design for ecosystem engineering while providing the baseline understanding needed for risk assessment and decision making.

Precise manipulation of natural or managed ecosystems can improve ecosystem resilience and productivity benefiting biosecurity and the bioeconomy. Successful, targeted ecosystem alterations are increasingly feasible by deliberately introducing nonnative or genetically modified plants and microbes. However, scientists currently lack the knowledge to successfully predict and introduce beneficial alterations, prevent undesired modifications, or predict the risks of proposed ecosystem biodesign efforts.

Today, microbes are routinely used as active ingredients in commercial biofertilizers and biopesticides to improve plant sustainability and productivity. However, these nonnative microbes often fail to establish and spread, requiring frequent reapplication. Successful establishment, dispersal, and beneficial impact of these microbes relies on the interaction of multiple phenotypic traits with the environment and resident microbial community. Identifying the genetic determinants of these complex traits requires a genome-wide interrogation of gene function. Moreover, the ability to monitor the movement, activity, and persistence of microbes in the environment is limited primarily to destructive approaches such as meta-sequencing technologies. Therefore, new techniques for *in situ* or nondestructive measurements and imaging of environmental microbial activities are needed to interrogate the dynamics of microbial invasions.

To this end, the project has developed a workflow to rapidly enhance transformation efficiency and genetic part characterization in nonmodel bacteria to engineer genome-wide libraries for high-throughput CRISPR interference (CRISPRi) screens. Researchers are developing biodesign tools for real-time *in situ* detection and quantification of microbial activity in ecosystems. These tools are currently being deployed in the plant growth–promoting bacteria *Bacillus velezensis*, a strong candidate for improving *Populus* sp. resistance to the pathogenic fungus *Sphaerulina musiva*.

First, the team is using CRISPRi to study how perturbation of gene expression impacts microbial establishment in the soil, rhizosphere, and *in planta*. Researchers built a 40,000-guide-RNA (gRNA) library targeting 10 gRNAs per annotated coding region in the genome and are performing growth assays to measure gRNA enrichment and depletion using next-generation sequencing under different selective conditions, such as during growth with root exudates and in different soil types. These functional assays will allow researchers to identify genetic perturbations affecting microbial establishment and inform engineering targets for future rhizosphere microbiome manipulation.

Second, the team is engineering a gas-based biosensor into *B. velezensis* and *S. musiva* to monitor the dispersal and activity of engineered strains belowground. These genetically engineered microorganisms will use an enzyme called methyl halide transferase to continuously produce methyl halide gas in vegetative cells. This indicator gas can be easily detected using gas chromatography–mass spectrometry without sample disruption from laboratory-to-field scales. Monitoring the location and activity of *B. velezensis* and *S. musiva* will aid in tracking and controlling the spread of microbes within and between select environments.

Collectively, these studies will provide new tools to study, engineer, and optimize targeted beneficial alterations to microbial communities in managed ecosystems.

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Advancing Towards Synthetic Biology that Can Detect and Control Plant–Fungal Interactions

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Project Goals: The Secure Ecosystem Engineering and Design (SEED) Science Focus Area (SFA) led by Oak Ridge National Laboratory combines unique resources and expertise in the biochemistry, genetics, and ecology of plant–microbe interactions with new approaches for analysis and manipulation of complex biological systems. The long-term objective is to develop a foundational understanding of how nonnative microorganisms establish, spread, and impact ecosystems critical to DOE missions. This knowledge will guide biosystems design for ecosystem engineering while providing the baseline understanding needed for risk assessment and decision making.

The introduction of microorganisms into new environments can have profound effects on resident communities (e.g., plants and associated microbiome) and local ecosystem services (e.g., soil stabilization and carbon sequestration). Depending on the organism and environmental context, these impacts can be positive, negative, or neutral. Over the last decade, the commercialization of several unique strains of beneficial fungi have begun improving agricultural yields at a lower cost-in-comparison to chemical fertilizers, while mitigating negative environmental impacts from agrochemicals. However, there are natural barriers limiting the use and reliability of beneficial fungi beyond the existing range of applications (i.e., host-specific benefits). Understanding these barriers will not only improve the ability to safely and reliably engineer ecosystems using fungi to reach specific goals (e.g., sustainable biofeedstocks) but also help predict and prevent economically and ecologically costly disease outbreaks. Evolutionary and ecological principles hindering targeted beneficial microbial inoculants frequently overlap with those overcome by invasive pathogens. Thus, learning about the first will enable better understanding of pathogen-mediated invasions.

Historically, there has been more research on the anthropogenic introduction and movement of fungal pathogens. In fact, recent population genomics analyses show human translocation of *Populus* across North America resulted in the spread of *Sphaerulina musiva* (formerly *Septoria musiva*) that now threatens natural forests and managed plantations. This pathogen has recently expanded to a new host, *P. balsamifera,* and causes fatal stem cankers in the DOE-flagship species *P. trichocarpa*.

Knowing the causal genetic factors associated with establishment and functional impact of *S. musiva* in the genus *Populus* will contribute to innovations in biodesign tools for early detection or altered outcomes of plant–fungal interactions. For the invader-centric research, researchers have assembled a pangenome from 146 *S. musiva* isolates collected from regions across North America spanning the range of several *Populus* species. This population genomics resource is being used to characterize the gene space of *S. musiva* and has identified more than 6 million single-nucleotide polymorphisms, of which 50% were not found in the reference genome. Researchers have performed genome-wide association studies for rapid genotype–phenotype discovery. Using the recently developed protoplast-mediated transformation system with CRISPR-Cas9, the team tested several useful biodesign targets for manipulating *S. musiva* virulence.

Understanding the establishment and spread of *S. musiva* must also consider the host genes that regulate plant–fungal symbiosis. In several instances, the team has demonstrated the role of G-type lectin receptor-like kinases (LecRLKs) in the susceptibility of a plant host to fungal colonization in both beneficial and pathogenic fungi. Building on this work, researchers hypothesized that advancing the understanding of G-type LecRLKs will inform biodesign strategies to detect and control plant–fungal interactions. To this end, researchers are working to determine how structural features of fungal cell walls are selectively recognized by G-type LecRLKs. This information is being used to design synthetic protein receptor systems that selectively detect fungal-derived ligands to report (biosensor) or permit (biocontrol) plant–fungal interactions.

Collectively, these studies will provide knowledge and tools to detect and control fungal invasions.

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Early Career

Engineering Continuous Trait Variation in Bioenergy Feedstocks to Optimize Growth on Marginal Lands

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Project Goals: As climate change progresses, bioenergy crops will need to withstand increasingly formidable water, nutrient, and temperature stresses. Though yields of C_4 grasses, such as *Sorghum bicolor,* have increased through breeding and improved agronomy, annual yield gains will be hard-hit by impending abiotic stressors (Prasad et al. 2021). Thus, new germplasm must be developed to maintain or continue to enhance yields. Unfortunately, relatively little is known about the traits that contribute to abiotic stress tolerance in sorghum or other related next-generation C_4 feedstock species. The project aims to develop a novel synthetic biology–based approach to determine the contribution of individual root features to abiotic stress tolerance. Synthetic genetic circuits will be employed to generate continuous variation in root depth and root branch density, so their contributions to stress tolerance can be studied with the ultimate goal of identifying optimums and generating more resilient plants.

Trait variation is key to understanding the contributions of specific plant features to environmental stress resilience. By measuring the fitness of plants with changes in a specific trait under different stress conditions, researchers can identify traits that are associated with greater resilience (Upadhyaya et al. 2016). The project is engineering sorghum plants to have variation in two key root traits—depth and branch density. The goal is to develop lines that dramatically reduce the number of plants that need to be phenotyped in gene-environment experiments in order to increase the number of abiotic stress conditions that can be tested simultaneously. The project's approach utilizes synthetic genetic circuits to tissue—specifically titrate—the expression of genes that control root development. Researchers have engineered Buffer gate components that can be used to vary gene expression over several orders of magnitude in C_4 grass protoplasts and are in the process of validating them in stably transformed lines. To tune root branch density, the team plans to express mutant auxin/indole-3-acetic acids (Aux/IAAs) at varying levels in specific root layers. The mutant Aux/IAAs should inhibit auxin response and prevent the development of lateral roots in a concentration dependent manner (Brophy et al. 2022). Researchers designed a library of mutant SbAux/IAAs with disruptions to their "degron" regions and have begun testing them in sorghum protoplasts (Moss et al. 2015). Initial results suggest that these mutant proteins are resistant to auxin-mediated degradation and can constitutively suppress the auxin transcriptional reporter DR5 (Yang et al. 2017). To modify root depth, the team is using CRISPR-Cas9 to knock out homologs of *DEEPER ROOTING 1 (DRO1)*—a gene identified in rice that alters root growth angles (Uga et al. 2013). Researchers are testing CRISPR guide RNA activity in sorghum protoplasts and plan to use the most efficacious guides for stable transformation. Once knocked out, *DRO1* will be reintroduced at a variety of expression levels using Buffer gates. This work is building toward a new approach for understanding the contribution of root architecture features to plant fitness.

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Moss, B. L., et al. 2015. "Rate Motifs Tune Auxin/Indole-3-Acetic Acid Degradation Dynamics," *Plant Physiology* **169**, 803–13.

Prasad, V. B. R., et al. 2021. "Drought and High Temperature Stress in Sorghum: Physiological, Genetic, and Molecular Insights and Breeding Approaches," *International Journal of Molecular Sciences* **22**, 9826.

Uga, Y., et al. 2013. "Control of Root System Architecture by DEEPER ROOTING 1 Increases Rice Yield Under Drought Conditions," *Nature Genetics* **45**, 1097–102.

Upadhyaya, H. D., et al. 2016. "Sorghum Germplasm Resources Characterization and Trait Mapping." In *The Sorghum Genome*, 77–94. Eds. Rakshit, S., and Y-H. Wang. Springer International Publishing, Cham. DOI:10.1007/978-3-319-47789-3_4.

Yang, J., et al. 2017. "Dynamic Regulation of Auxin Response during Rice Development Revealed by Newly Established Hormone Biosensor Markers," *Frontiers in Plant Science* **8**.

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Lipid Membrane Biology of Microbial Cell Factories During Microaerobic Fermentation

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Project Goals: The goal of this Early Career Program project is to engineer the structure and properties of cell membranes to improve the performance of industrially relevant microbes. The project's first objective is to enhance the rate and efficiency of the respiratory metabolism by engineering the organization of the electron transport chain. Engineering efforts will define the limits of respiratory metabolism and seek to increase the production of energy-intensive next-generation biofuels. The second objective is to apply the emerging biochemistry of intracellular lipid trafficking pathways to develop new transporters for the capture of valuable biochemicals produced by the engineered yeast.

The project will present advancements in three areas of lipid membrane biology relevant for the performance of yeast microbial cell factories. In the first direction, the team has elucidated key factors that allow for mitochondrial function in fermentation conditions that are characterized by low oxygen availability. Paradoxically, mitochondria proliferate under these conditions, and the inner mitochondrial membrane increases its surface area and complexity. Researchers have found that synthesis and remodeling of the tetra-acyl lipid cardiolipin is essential under microaerobic fermentation due to lipidomic changes resulting from the loss of oxygen-dependent desaturase activity. In the second direction, researchers have characterized a putative lipid transfer protein (LTP) that is predicted to bind squalene, a biolubricant and intermediate in ergosterol biosynthesis. Researchers have found that loss of this LTP, Sfh2, results in accumulation of squalene under microaerobic conditions. The team is currently testing if this LTP traffics squalene from its site of synthesis in the endoplasmic reticulum to storage sites in lipid droplets *in vivo*. The project is also testing its *in vitro* activity and proposes that it could be harnessed to better extract squalene from cell factories. In the third direction, researchers have engineered sterol metabolism in yeast to develop cells that better tolerate high temperature and low oxygen fermentations. The project envisions these strains as allowing for new bioproduction capacities outside of standard conditions.

Venkatraman, K., et al. 2023. "Cristae Formation is a Mechanical Buckling Event Controlled by the Inner Mitochondrial Membrane Lipidome," *The EMBO Journal*. DOI:10.15252/embj.2023114054.

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Systems Metabolic Engineering of *Novosphingobium aromaticivorans* for Lignin Valorization

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Project Goals: The goal of this project is to engineer a nonmodel bacterium, *Novosphingobium aromaticivorans*, for valorization of depolymerized lignin to value-added bioproducts. The project involves (1) discovery and optimization of pathways for assimilation of lignin-derived aromatic compounds, (2) engineering conversion pathways that match the stoichiometry of aromatic catabolism, and (3) development of genome-scale mapping techniques to identify new engineering targets in nonmodel bacteria.

Lignin is one of the abundant renewable materials found in nature. This heterogeneous aromatic polymer is composed of a variety of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monomers that are connected by diverse chemical linkages. Lignin valorization would improve biofuel economics, for example, through bacterial conversion of thermochemically depolymerized lignin into valuable bioproducts. *N. aromaticivorans* F199 is an Alphaproteobacterium capable of degrading G, S, and H monomers and, due to its genetic tractability and broad catabolic capabilities, is an emerging model organism for conversion of lignin-derived aromatic compounds. However, F199 cannot natively catabolize every component of depolymerized lignin, which limits conversion yields (Azubuike et al. 2022).

Researchers are identifying new aromatic degradation pathways to further increase the catabolic potential of F199 using a combination of barcoded transposon insertion sequencing, proteomics, experimental evolution, and *in vitro* biochemistry. The team demonstrated this approach with the aromatic monomer syringate, the β-1 linked dimer 1,2-diguaiacylpropane-1,3-diol (DGPD), and, more

recently, the monomer guaiacol (Bleem et al. 2022; Cecil et al. 2018; Presley et al. 2021). However, there are multiple lignin-derived aromatic compounds that F199 catabolizes poorly or not at all. Researchers have evolved F199 to rapidly and completely catabolize the common β-O-4 dimer guaiacylglycerol-β-guaiacyl ether (GGE) and, in the process, identified an uncharacterized native catabolic pathway for the monomeric intermediate β-hydroxypropiovanillone. Researchers have also isolated a *Novosphingobium* strain that can assimilate the β-β linked dimer pinoresinol and fully characterized the pinoresinol catabolic pathway. Current efforts focus on transfer of heterologous catabolic pathways into F199.

In addition to optimizing lignin assimilation, researchers are converting the resulting intermediates into value-added products, such as building blocks for bioderived polymers. Using a combination of heterologous pathway expression, experimental evolution, and targeted chromosomal modification, researchers have enabled and improved conversion in F199 of the model lignin-derived aromatic substrate ferulate into 5-aminovaleric acid (5-AVA). Degradation pathways for 5-AVA have also been identified in F199 and are being removed. Additional optimization targets have been identified through metabolomic analysis of wildtype and engineered strains.

Finally, to better understand the effects of host genetic variation on pathway function, researchers are adapting a novel technique, bacterial quantitative trait locus (QTL) mapping, to F199. Researchers have demonstrated directional intraspecific recombination between strains of *N. aromaticivorans* driven by an integrative and conjugative element (ICE) in the donor strain. The team is currently identifying the origin of transfer of this ICE to improve transfer. By combining novel pathway discovery, heterologous expression, and genome-scale optimization, researchers are engineering *N. aromaticivorans* F199 to efficiently valorize lignin-derived compounds.

Azubuike, C. C., et al. 2022. "Microbial Assimilation of Lignin-Derived Aromatic Compounds and Conversion to Value-Added Products," *Current Opinion in Microbiology* **65**, 64–72.

Bleem, A. et al. 2022. "Discovery, Characterization, and Metabolic Engineering of Rieske Non-Heme Iron Monooxygenases for Guaiacol O-Demethylation," *Chem Catalysis* **2**, 1989–2011.

Cecil, J. H., et al. 2018. "Parallel Identification of Catabolism Pathways of Lignin-Derived Aromatic Compounds in Novosphingobium aromaticivorans," *Applied and Environmental Microbiology* **84**, AEM.01185-18.

Presley, G. N. et al. 2021. "Pathway Discovery and Engineering for Cleavage of A B-1 Lignin-Derived Biaryl Compound," *Metabolic Engineering* **65**, 1–10.

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Encapsulin Nanocompartment Systems in *Rhodococcus opacus* for Compartmentalized Biosynthesis Applications

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Project Goals: This project is focused on understanding how encapsulin nanocompartment systems can be used to enhance the biosynthesis of next-generation biomaterials in *Rhodococcus* species. The project seeks (1) to probe the mechanistic basis for how these compartments are regulated, biosynthesized, and maintained and (2) engineer these systems to achieve new biosynthetic functions.

With recent innovations in synthetic biology, engineered microbes now have the potential to produce a wide variety of bioproducts from renewable sources (e.g., biomass) to support the U.S. bioeconomy. Biosynthetic pathways leading to these products are often hindered by poor reaction efficiencies and toxicity, however, resulting in low yields and impure products. Compartmentalization of these pathways has the potential to overcome these challenges through colocalization, concentration, and sequestration. The goal of this early career research is to identify mechanisms for engineering compartmentalized biosynthesis in the emerging model bioproduction bacterium, *Rhodococcus opacus* PD630, using its native encapsulin nanocompartment system (herein called encapsulins). Toward this goal, the group have integrated a fluorescent reporter into the *R. opacus* genome under the control of the native encapsulin promoter. With this, the group is investigating the regulation, biosynthesis, and maintenance of the native encapsulin system using growth assays and transposon mutagenesis.

These studies will uncover the native pathways that govern encapsulin synthesis with the goal of ultimately harnessing these pathways for improved recombinant encapsulin

formation and yield. The group is also developing novel, high-throughput methods for the engineering of encapsulins to systematically identify optimal insertion locations and sequences, as well as easily modulate their properties. This will enable researchers to quickly tailor the properties of encapsulins to process requirements, greatly enhancing the utility of this system. As a case study, the *R. opacus* encapsulin system will be redirected to support and control the biosynthesis of cadmium sulfide nanoparticles, semiconducting materials used in optical and electronic applications. Ultimately, this work will establish encapsulin compartmentalization systems as a means of improving yields and enabling new biosynthetic routes toward next-generation bioproducts and biomaterials in support of the DOE's mission to build a strong bioeconomy.

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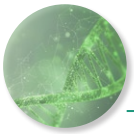

National Laboratory Projects

Discovery and Functional Characterization of Genomic Islands for Nonmodel Bacterial Systems

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The Intrinsic Control for Genome and Transcriptome Editing in Communities (InCoGenTEC) Science Focus Area aims to develop strategies for biocontainment, enable safe transformation of nonmodel prokaryotes using phage vectors, and understand gene mobility in microbial communities. This project's overall goals are to mechanistically understand gene mobility events through comprehensive computational mapping of integrase and transposon-driven mobility, perform functional genomics studies to identify genes and pathways responsible for mobility, identify novel genes for use in biocontainment mechanisms, and utilize the prophages from the genomic island (GI) database to transform nonmodel microbes towards the goal of safe microbial community transformation.

Development of phage vectors requires closing two major knowledge gaps: (1) idenifying viable phages that infect nonmodel bacterial species and (2) functionally understanding these phages.

Researchers have three strategies to obtain viable phages for nonmodel bacteria: environmental isolation, prophage induction, and synthetic phage rebooting. The team used environmental isolation in low- or high-throughput to isolate phages from environmental reservoirs. Two new environmental phages, each with unique features, were isolated from soil using traditional low-throughput methods. *Pseudomonas pudita* phage MiCath contains an entire queuosine biosynthesis cassette to produce modified nucleotides protecting the phage genome from nuclease activity, and *Rhodococcus* phage Perlina has three tRNA genes and a split lysin gene.

To speed up the discovery of novel phages, researchers have developed a high-throughput phage isolation method (HtPIP) that enables a single soil sample to be used for screening of up to 96 strains. Researchers are currently using HtPIP to isolate a number of phages for a diverse range of bacterial isolates from soil microbial communities.

Experimental phage hunting (even in high throughput) is slower than discovery of prophages from bacterial genomic sequence data. The team has developed a genomic island database, which includes precisely defined prophage genomes that can be "mined" for any bacterial species of interest (Mageeney et al. 2020). This database contains ~20x more phages that currently found in public phage sequence repositories. Practically, however, many of the strains that harbor these prophages are inaccessible. Researchers have developed a system to synthetically rebuild and reboot these prophages from sequence alone using DNA synthesis, yeast-assisted assembly, and cell-based phage production.

To understand the phage biology and engineering constraints, and to harvest useful gene products for delivery and transformation of synthetic genetic elements, researchers must understand the functions of genes contained within the phages and genomic islands. The team plans to use CRISPRi, CRISPRi-ART, and DART technologies to enable functional genomics for the nonmodel bacteria and

their mobile genetic elements. Using these CRISPR tools, researchers will perform whole-MGE loss-of-function screens to identify candidate genes for removal or reuse. Overall, this work provides a foundation for understanding genomic islands, allows informed design of phages for vectors, and greatly increases the ability to mine prophages.

Mageeney, C. M., et al. 2020. "New Candidates for Regulated Gene Integrity Revealed Through Precise Mapping of Integrative Genetic Elements," *Nucleic Acids Research* **48**(8), 4052–65.

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Renewed Utility of Tyrosine Integrases

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The Intrinsic Control for Genome and Transcriptome Editing in Communities (InCoGenTEC) Science Focus Area aims to develop strategies for biocontainment, enable safe transformation of nonmodel prokaryotes using phage vectors, and understand gene mobility in microbial communities. The overall project goals are to: (1) mechanistically understand gene mobility events through comprehensive computational mapping of integrase- and transposon-driven mobility; (2) perform functional genomics studies to identify genes and pathways responsible for mobility and identify novel genes for use in biocontainment mechanisms; and (3) utilize prophages from a genomic island database to transform nonmodel microbes toward the goal of safe microbial community transformation.

DNA integrases catalyze recombination between specific attachment (att) sites on two circular DNAs: the attB site on a bacterial chromosome and the attP site on a pre-island circle. This results in a single circle with the genomic island precisely integrated into the chromosome. Integrases retain all DNA strands until after recombination is complete, forming covalent intermediates at either a catalytic tyrosine or serine residue depending on the protein family. If an att site is available for use in a locus, this reaction mechanism can provide an inherently more efficient and safer approach to genome editing than CRISPR methods which first introduce a double-stranded break in the chromosome. Additional safety and control come from the property of directionality: integrases require an additional partner protein (e.g., excisionase or recombination directionality factor) to catalyze the reverse excision reaction but not the forward integration reaction.

Serine integrases have been favored for genome editing applications because tyrosine integrases are perceived to require additional protein factors from their bacterial hosts. This dependence on host factors, particularly the integration host factor (IHF), occurs for certain classical tyrosine integrases (e.g., phage lambda) but may not apply to integrases from the many bacterial phyla that do not harbor IHF genes, nor to all integrases from IHF+ species. Using project software to precisely map hundreds of thousands of tyrosine integrase att sites, the research team assembled a panel of diverse tyrosine integrases which were assayed using *E. coli*-based *in vivo* and cell-free assays. Many integrases from phyla not known to bear IHF genes were functional, even in IHF-deficient genetic backgrounds, and in cell-free assays where IHF was diluted ~10-fold.

This work demonstrates that bias against tyrosine integrases has resulted from a misperception; most are not dependent on host factors. Tyrosine integrases are ~8-fold more abundant than serine integrases, offering far more site-specificity. Vetting numerous tyrosine integrases by assay, with diverse site-specifities, is expected to expand safe gene editing biotechnology.

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ENVIRONMENTAL
MICROBIOME

Conversion of Lignocellulosic Plant Biomass into Industrial Chemicals via Metabolic Engineering of Two Extreme Thermophiles, *Caldicellulosiruptor bescii* and *Pyrococcus furiosus*

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Project Goals: This project aims to metabolically engineer two extreme thermophiles, *Caldicellulosiruptor bescii* (T_{max} 90°C) and *Pyrococcus furiosus* (T_{max} 103°C), for the renewable production of key industrial chemicals through the conversion of lignocellulosic biomass, with targets including acetone, 2,3-butanediol, 1-propanol, 3-hydroxypropionate, and ethanol. This work includes efforts of carbon and energy optimization through harnessing carbon dioxide (CO_2) and dihydrogen (H_2) produced from fermentation into desired products and energy, respectively. Select enzymes responsible for degradation of lignocellulose will be expressed in *P. furiosus* to allow growth on cellulose and xylan. System-wide metabolic and regulatory models for both organisms will be leveraged to optimize biomass degradation and product yield for the target chemicals.

Extremely thermophilic organisms present a valuable opportunity to convert lignocellulosic biomass to industrial chemicals, as conversion at high temperatures offers specific advantages, such as reduced contamination risk and temperature-dependent separation of volatile products. *P. furiosus* is a hyperthermophilic archaeon (T_{opt} 100°C) with a growth range from 70°C to 103°C. The research group seeks to harness this organism's extreme thermophily and robust genetic system for the production of chemicals of interest. *P. furiosus* has been previously engineered to produce lactate, ethanol, 3-hydroxypropionate, acetoin, and butanol. The *P. furiosus* alcohol dehydrogenase F (AdhF) was recently identified as the ethanol-forming enzyme, with AdhF overexpression resulting in increased ethanol yields at temperatures up to 95°C (Lipscomb et al. 2023). *P. furiosus* strains producing 1-propanol were recently developed by constructing a nine-enzyme pathway consisting of both heterologous and native enzymes, although the characterization of these strains is still underway. Additionally, efforts are underway to express hemicellulases and cellulases in *P. furiosus*, with the goal of enabling the organism to grow directly on xylan and cellulose, as *C. bescii* does natively.

The other subject of this work, *C. bescii,* has been metabolically engineered for the production of acetone, ethanol, and other various alcohols. Recent work engineered 2,3-butanediol production in *C. bescii* when grown on unpretreated biomass (Tanwee et al. 2023). To further the researchers' understanding of *C. bescii* and related thermophiles, this team has sequenced the genomes of many species in the genera *Caldicellulosiruptor, Thermoclostridium,* and *Thermoanaerobacter* (Bing et al. 2023a; Bing et al. 2023b; Manesh et al. 2024), leading to a reassessment of the taxonomic classification for the genus *Caldicellulosiruptor* and the order Thermoanerobacterales (Bing et al. 2023c). To better understand the ability of *C. bescii* to degrade biomass, the presence of microorganisms indigenous to various types of biomass was explored, alongside work to better understand cell–substrate associations during biomass solubilization (Bing et al. 2023d; Laemthong 2023). Work is also ongoing to engineer the cytoplasmic hydrogenase from *P. furiosus* into *C. bescii* to provide redox balancing for pathways dependent on the production of nicotinamide adenine dinucleotide phosphate (NADPH). System-wide metabolic and regulatory models of both *C. bescii* and *P. furiosus* have been created; these models have been and are currently being harnessed to predict optimization approaches for biomass conversion and product formation (Rodionov et al. 2021; Zhang et al. 2021; Vailionis 2023).

Bing, R. G., et al. 2023a. "Complete Genome Sequences of *Caldicellulosiruptor acetigenus* DSM 7040, *Caldicellulosiruptor morganii* DSM 8990 (RT8.B8), and *Caldicellulosiruptor naganoensis* DSM 8991 (NA10)," *Microbiology Resource Announcements* **12**(3). DOI:10.1128/mra.01292-22.

Bing, R. G., et al. 2023b. "Complete Genome Sequences of Two Thermophilic Indigenous Bacteria Isolated from Wheat Straw, *Thermoclostridium stercorarium* subsp. Strain RKWS1 and *Thermoanaerobacter* sp. Strain RKWS2," *Microbiology Resource Announcements* **12**(3). DOI:10.1128/mra.01193-22.

Bing, R. G., et al. 2023c. "Whither the Genus *Caldicellulosiruptor* and the Order Thermoanaerobacterales: Phylogeny, Taxonomy, Ecology, and Phenotype," *Frontiers in Microbiology* **14**, L1212538. DOI:10.3389/fmicb.2023.1212538.

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Lipscomb, G. L., et al. 2023. "Manipulating Fermentation Pathways in the Hyperthermophilic Archaeon *Pyrococcus furiosus* for Ethanol Production up to 95°C Driven by Carbon Monoxide Oxidation," *Applied and Environmental Microbiology* **89**(6). DOI:10.1128/aem.00012-23.

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Rodionov, D. A., et al. 2021. "Transcriptional Regulation of Plant Biomass Degradation and Carbohydrate Utilization Genes in the Extreme Thermophile *Caldicellulosiruptor bescii*," *mSystems* **6**(3), e01345-20. DOI:10.1128/mSystems.01345-20.

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Zhang, K., et al. 2021. "Genome-Scale Metabolic Model of *Caldicellulosiruptor bescii* Reveals Optimal Metabolic Engineering Strategies for Biobased Chemical Production," *mSystems* **6**(3), e0135120. DOI:10.1128/msystems.01351-20.

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How Microbes and Minerals Make Necromass that Persists

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Project Goals: Most of the Earth's terrestrial carbon is stored in soil organic matter (SOM), and new SOM is derived largely from microbial necromass (Liang and Balser 2011). Necromass forms when microbes produce extracellular compounds, like extracellular polysaccharides and enzymes, or succumb to environmental stress and lyse. Because most soil microbes live attached to surfaces, necromass tends to remain associated with minerals, eventually persisting as SOM (Creamer et al. 2019). New necromass is the most sensitive to decomposition (Dong et al. 2021), so understanding new necromass decomposition directly informs long-term SOM stability and persistence.

Including microbial parameters in ecosystem models improves projections of soil carbon (C) stocks (Wieder et al. 2015). Soils with large C stores generally have large and active microbiomes (Liang et al. 2019), suggesting that yield or turnover drives necromass formation and persistence. However, growth is constrained by the acquisition of limiting resources. Soil microbes are primarily C limited, but soil minerals affect nutrient limitation, in turn affecting the ability to make new extracellular enzymes or dictating the size of cells and even genomes (Sorensen et al. 2019). Stress can also divert resources away from growth and nutrient acquisition, altering necromass deposition and microbial biomass composition (Fernandez and Kennedy 2018).

Mineral–organic associations are the most quantitatively important C storage mechanism in soils (Oldfield et al. 2018). Soil minerals modulate availability of energy and nutrients to microbes by restricting water and oxygen flow (Keiluweit et al. 2017). Clay minerals also slow microbial growth by sorbing added exogenous substrates (Finley et al. 2021). Both soil pore size and clay composition are intertwined with microbial traits to regulate growth and nutrient acquisition. By exploring traits associated with growth and resource acquisition from necromass biomolecules in soils and soil communities, researchers can better predict necromass persistence.

The goal is to better define the interactive roles of soil mineralogy and microbiomes that contribute to the persistence of necromass as SOM. The project's overarching hypothesis is that necromass biomolecules are sensitive to decomposition

depending on microbiome traits, nutrient bioavailability, and soil types. This work has four specific objectives:

- Define microbial populations capable of degrading necromass macromolecules.
- Define the mineral characteristics and microbial traits that drive necromass decomposition.
- Define the food webs and fates of necromass macromolecules in soils over time.
- Model the microbial or metabolic traits that best predict necromass formation.

Three experiments explore how microbes and minerals make persistent necromass, followed by statistical and mechanistic modeling to define conditions conducive to persistent necromass.

Some soil C persists, sometimes for a long time, but scientists don't know enough about this process to predict the long-term storage of C. New organic matter enters soil through plant detritus, and similar amounts enter soil through the continuous recycling of nutrients by microbial turnover, which generates microbial necromass. This microbial necromass represents a steady stream of new organic matter to soil, but new necromass is very sensitive to decomposition and loss as carbon dioxide (CO_2). Surface attachment to minerals and other organic matter is the prevailing theory on the mechanism of soil C stabilization. However, reactive surfaces and soil pores reduce the bioavailability of nutrients that drive microbial turnover. How do microbes and minerals make necromass that persists?

Here, the project shows preliminary results from an ongoing experiment that assesses the impacts of soil pore size and clay activity on the formation and cycling of necromass. The team has established nine artificial soils that consist of 90% acid-washed sand and 10% clay, which vary in both pore size (25 to 45, 75 to 105, and 150 to 250 micrometer particle sizes, using silica quartz sand) and clay activity (kaolinite and montmorillonite in 1:1, 1:9, and 9:1 mass ratios). These model soils were inoculated with soil microbes extracted from a temperate deciduous forest soil (Harvard Forest, Petersham, Mass.) and have been fed weekly since February 2023 with 0.5 milligram cellobiose C per gram of soil and 0.05 mg ammonium nitrate nitrogen per gram of soil and maintained at 45% soil moisture.

Preliminary results show that soils with smaller particle sizes and more active clays differ in microbial biomass (DNA yields), community composition (amplicon sequencing), and activity (CO_2 production) compared to coarser-textured soils with less active clays. The team additionally presents an upcoming experiment that incubates the mature model soils with representative necromass biomolecules (carbohydrates, proteins, nucleic acids, and lipids). Researchers will use oxygen-18 (^{18}O) water to follow ^{18}O incorporation into proteins in response to each necromass biomolecule. A novel combination of stable isotope probing–metaproteomics and metagenomics can be used to define the metabolic pathways and microbial populations active across soil and macromolecule types, as well as substrate-specific C use efficiency. Additionally, the team will track the fate of necromass C through continuous monitoring of CO_2 production and assessment necromass C stocks in microbial biomass (via chloroform fumigation) and particulate versus mineral-associated pools (via density fractionation).

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Dead or (Still Not) Alive: Patterns and Drivers of Soil Viral Activity, Turnover, and Decay

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Project Goals: The overarching goal of this project is to assess and compare the contributions of active, infectious viruses and inert viral particles to biogeochemistry across diverse terrestrial ecosystems. Using a multiomics approach, researchers seek to establish spatiotemporal patterns in soil viral community composition and activity linked to host carbon and nitrogen metabolism in grasslands, shrublands, woodlands, and wetlands. Through laboratory experiments, researchers are investigating the chemical composition, fate, transport, and integrity of viral particles in soil. By integrating field and laboratory experiments across a variety of soil edaphic properties and spatiotemporal scales, this project is expanding the understanding of the soil virosphere and its influence on carbon and nutrient cycling.

Viruses are highly abundant but poorly characterized members of the soil microbiome. By infecting soil microbes, viruses likely have substantial impacts on terrestrial biogeochemical processes under their hosts' control. Viral particles (virions) may also play more direct roles in soil biogeochemical cycling as packets of carbon, nitrogen, and phosphorus, but the timescales and environmental conditions that determine virion infectivity, transport, and sorption to soil particles are unknown. This project uses field, laboratory, and computational approaches to distinguish between infective and inert virions and to assess their contributions to soil biogeochemical cycling.

Using a <0.22 μm viral size-fraction metagenomics (viromics) approach, researchers are exploring the conditions and temporal scales over which virions are produced, remain infective, and decay in soil. Researchers are also tweaking and rigorously testing the viromics protocol to facilitate access to different parts of the soil virosphere, including decayed viral particles and virions with different chemical compositions. Researchers find that soil viromes usually capture very recently active viral communities, but they can be dominated by decayed viral particles after extreme temperature perturbations and in seasonally dry soils. For example, heating experiments revealed that virion survival thresholds are similar to those of bacteria, with a reduction in survival at 60°C and nearly complete removal of intact virions at 90°C.

Since the viromics method relies on extraction buffers to desorb virions from the soil matrix prior to DNA extraction, extraction efficiency and the composition of the recovered viral community could be affected by interactions among virions, soil, and the chosen extraction buffer. A test of seven buffer chemistries revealed that recovered viral communities were largely unchanged by buffer chemistry. Rather, viral communities differed most significantly by soil type (forest, grassland, or wetland) and differed more significantly just ~1 m apart in the field than by extraction buffer chemistry. Thus, extraction buffer chemistry is likely of much lower importance than ecological considerations, such as spatial distance, in the design of future soil viral ecological studies.

Centered on a highly spatiotemporally resolved viromic study of two habitats in the Jepson Prairie grassland (eight locations, 29 timepoints from November 2020 to February 2022), the ongoing analyses of >300 viromes seek to unravel the relative contributions of space, time, habitat, and dispersal on soil viral community composition. Briefly, viromes were most distinct by habitat (between mounds and their adjacent swales, defined by differences in topography, plant communities, and hydrology), but each habitat exhibited different patterns over time. Although cumulative viral community richness continued to increase over the multi-year study period, prokaryotic community richness showed signs of leveling off, suggesting much greater viral than prokaryotic diversity.

In sum, the factors that structure soil viral communities differ substantially across scales and habitat characteristics, and a better understanding of these processes will enable more robust predictions of viral contributions to terrestrial biogeochemical cycling.

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Pyrocosms to Measure Influence of Fire Intensity, Time, and Soil Depth on Microbial Succession, Cross-Kingdom Interactions, and Greenhouse Gas Emissions

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Project Goals: Wildfires are increasing in frequency, size, and severity across the globe. Unlike ecosystem disturbances that primarily impact vegetation, wildfires kill microbes, thereby dramatically altering the composition, function, and abundance of post-fire soil microbiomes, with downstream impacts on soil nitrogen (N) cycling. Despite widespread microbial mortality during fires, post-fire environments can also favor the growth of pyrophilous, fire-loving, microbes. Pyrophilous microbes have been documented in widespread post-fire habitats, yet their traits and impacts on ecosystem N losses remain largely uncharacterized. Here, researchers focus on how wildfire severity and pyrophilous microbial interactions regulate N cycling and the emission of greenhouse gases like nitrous oxide (N_2O), a powerful greenhouse gas with 300 times the warming potential of carbon dioxide, with implications for long-term ecosystem recovery, regional air quality, and Earth's climate. The overarching project goal is to answer the question: do conserved genomic traits and cross-kingdom interactions drive post-fire N cycling across ecosystems?

Understanding how soil microbial interactions and traits govern N cycling is critical to forecasting post-fire soil N dynamics and ecosystem recovery. Using pyrophilous microbiomes as model systems, researchers will scale up across systems of increasing complexity, from individual genomes to more complex microbiomes, allowing researchers to predict the impacts of wildfire disturbance on ecosystem N cycling with the DEcomposition Model of ENzymatic Traits (DEMENT). Across three ecosystems that are experiencing increased fire frequency (Mediterranean grasslands, chaparral shrublands and montane coniferous forests) researchers ask: (1) how do microbial traits change during post-fire succession?; (2) how does fire severity influence microbial succession and gene expression of N cycling functions?; (3) how do cross-kingdom interactions change during post-fire succession?; and (4) how do traits and interactions affect ecosystem N fates and cycling?

To answer these questions, researchers are using experimental pyrocosms to simulate soil heating under controlled and replicable conditions. These pyrocosms will enable researchers to test how microbial succession and cross-kingdom interactions affect N cycling and greenhouse gas emissions under varying fire severities, biomes, and soil depths. In fall 2023, the team burned soils collected from a Southern California Chaparral shrubland within the Cleveland National Forest adjacent to the 2018 Holy Fire burn scar where researchers have studied natural microbial succession post-fire. The team used three treatments (control, low-, and high-intensity fires) and sampled at seven timepoints ranging from pre-fire to 4 months post-fire (burns occurred on September 5, 2023, and the last sampling date was January 5, 2024). The experimental replicates yielded highly similar temperature profiles within treatments, with peak temperatures much higher in the high- than low-intensity treatments, and temperatures declining as expected with depth.

The team also succeeded in achieving very different soil surface temperatures for high- and low-intensity treatments that approximated natural fire conditions with high-intensity treatments nearing 600°C and low intensity around 300°C. Temperatures declined linearly from surface to 10cm below the surface with very little variation between replicates.

So far, researchers have collected soils from two depths (5 and 10 cm below surface) at seven time points from pre-fire to 4 months post-fire. Researchers have extracted DNA from all samples and conducted 16S and internal transcribed spacer polymerase chain reactions and are awaiting Illumina MiSeq data detailing how bacterial and fungal richness changed over time post-fire. Researchers have also extracted viromic DNA and RNA from all samples and have submitted them for sequencing to determine how bacterial, fungal, and viral communities changed across time and depth after high- and low-intensity chaparral fires. Finally, the team performed extensive biogeochemical analyses to determine how N cycling and greenhouse gas emissions are impacted by fire intensity and time. Once the amplicon sequencing data is analyzed, researchers will select a subset to perform metagenomics and metatranscriptomics to determine how gene abundances and transcription change across time and fire severity treatments.

Burning pyrocosms of sieved Holy Fire soil increased soil pH from 6.2 ± 0.0 to 7.0 ± 0.2 and increased potassium chloride–extractable ammonium (NH_4^+) content from 2.7 ± 0.1 μg N/g soil to 48.2 ± 5.8 μg N/g soil 1 week after the burn in the top 10 cm of soil. Researchers also found that both soil pH and NH_4^+ content were positively correlated with peak burn temperatures. NH_4^+ content decreased as nitrate increased more rapidly in the low-burn treatment than the high-burn or control treatments during the 4 months after burning, suggesting that low-intensity wildfires

may fuel high nitrification rates in chaparral soils. The team conducted high-resolution N_2O, carbon dioxide, and nitrogen oxide gas flux and isotope measurements for all seven timepoints and are currently processing these data.

The next goal is to burn a second round of pyrocosms at University of California–Riverside using soils collected from Southern California grasslands. The team has received the permit to collect the soil and will be burning grassland pyrocosms in spring 2024. This will allow researchers to compare how microbial succession, cross-kingdom interactions, and greenhouse gas emissions are altered across fire severity and soil depth in two fire-prone dryland ecosystems.

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Applying Metabolic Models to Mechanistically Understand and Predict Interactions Between Anaerobic Methanotrophic Archaea and Sulfate-Reducing Bacteria Strains in Geochemical Cycling Processes

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Project Goals:

- Investigate metabolic syntropy between anaerobic methane oxidation (AOM) archaea with sulfate-reducing bacteria (SRB).
- Design a coupled methane (archaea)/sulfate electron transport chain (ETC) model.
- Evaluate the interaction between diverse strains of AOM archaea and SRB.

Microbial communities of anaerobic methanotrophic (ANME) archaea and SRB annually prevent the release of gigatons of methane into the environment and are therefore critical agents in climate regulation and geochemical cycling. The "reverse methanogenesis" of methane oxidation in ANME, which requires electron transfer to a syntrophic partner, is the proposed syntrophic mechanism that drives sulfate-coupled anaerobic oxidation of methane in these communities; however, their physiology, interactions, and ecology remain opaque.

To model this metabolic system and resolve mechanistic details, the research team improved its reconstruction pipeline for all archaea and bacteria to construct genome-scale metabolic models: the ModelSEED2. The new archaea pipeline now captures unique pathways and reaction intermediates to archaea. Researchers concurrently developed a suite of community modeling tools to mechanistically simulate syntrophic interactions within this community under native conditions, which is essential to contextualize the ecological roles of ANME and SRB. These community modeling tools permit the parameterization of omics data that represent metabolic phenotypes. This method allows researchers to better recapitulate community dynamics with thermodynamic and uptake constraints. Further, the group additionally developed new tools to leverage pangenome information from phylogenetically close strains to improve model reconstruction for metagenome-assembled genomes (MAGs), which are often incomplete when analyzed on their own. These tools are critical for modeling ANME strains because they cannot be isolated in the laboratory and are thus all MAGs. Due to the limited biomass available in these systems, ANME MAGs are also often incomplete. To overcome this challenge, group members applied a pangenome-based approach to enhance their ANME MAG models to include all core genes from the pangenome, boosting the size of their ANME models by hundreds of conserved reactions while still preserving the distinctive metabolic features that distinguish each ANME clade. Researchers constructed metabolic models of several ANME and SRB MAGs that were assembled and binned from metagenomic data from previous studies (Chadwick et al. 2022; Murali et al. 2023). The group implemented an energy metabolism pathway that couples anaerobic methane oxidation with the sulfate reduction pathway.

The improved annotation accuracy of these models will empower community simulations towards resolving the reverse methanogenesis hypothesis, which may explain the natural stability and selectivity of these communities and would ultimately clarify anthropogenic influences on these keystone communities and biogeochemical cycles in marine environments. The group performs a detailed accounting for the flow of nutrients and energy within its community model to mechanistically explain low yields and slow growth in these systems.

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Legacy Effects of Warming Alter Simple and Complex Soil Organic Matter Decomposition

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Project Goals: Microorganisms are major engines of the land carbon cycle, responsible for influencing the composition and radiative properties of the atmosphere, and for both creating and consuming soil organic carbon, a resource that provides multiple ecosystem services, and, when lost, exacerbates climate change. The project investigates the interactions within microbial communities and between microbes and their environment that underpin these dual roles of microorganisms in creating and consuming soil carbon. An overarching objective is to develop and apply omics approaches to investigate microbial community processes involved in carbon and nutrient cycling; interrogating community and taxon-specific microbial controls over key biogeochemical processes in terrestrial environments and testing quantitative ecological and biogeochemical principles using omics data. This work aims to facilitate the scaling of taxon-specific microbial data to connect the ecology of microorganisms with ecosystem-level rates of carbon and nutrient cycling.

Many ecosystems are predicted to become warmer and drier as global change progresses. Since carbon cycling feedbacks influence climate, understanding how warmer and drier conditions affect microbial interactions that influence the cycling and storage of carbon in terrestrial ecosystems is critical. However, researchers currently lack a holistic understanding of how interactions within soil microbial communities are impacted by global change, limiting the ability to understand and predict soil carbon cycling. Here, researchers aimed to understand how antagonistic and mutualistic microbial interactions are impacted by long-term warming and related to changes in carbon cycling. To address this aim, researchers leveraged a long-term experiment that was established in the San Francisco Peaks region near Flagstaff, Arizona, in 2002. Collared soil peds from a mixed conifer forest were either transplanted to a lower elevation (ponderosa pine forest) or incubated in their home ecosystem. This relocation of soil served as a proxy for climate change as the ponderosa pine ecosystem is ~2°C warmer. To test how climate change impacts soil organic carbon cycling via microbial interactions, ambient and warmed soils were harvested after 21 years and incubated in the laboratory at a uniform temperature and moisture. Isotopically labeled (^{13}C) organic matter substrates of varying chemical complexity were added to the soil to observe microbial processing of complex (plant litter) and simple (synthetic root exudates) organic carbon substrates. Preliminary results indicate that a legacy of warming causes soil microbes to mineralize simple carbon (synthetic root exudates) more rapidly but have a reduced ability to degrade complex leaf litter. As complex organic substrates are often decomposed by a consortium of microorganisms working in concert, reduced litter decomposition could result from a warming-induced weakening of mutualistic microbial interactions. Future work will investigate these community dynamics using molecular and biogeochemical techniques. Specifically, quantitative stable isotope probing (qSIP) will be used to identify the microbial taxa that shift their carbon assimilation under warming, and paired with metagenomics, transcriptomics, and metabolomics to determine how microbial interactions govern ecosystem responses to global change. The overarching goal of this research is to understand how climate change alters soil biogeochemistry and carbon sequestration potential via changes in the microbial interactions that govern decomposition.

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Friends and Foes: How Microbial Predators Influence Nutrient Cycling in Soil

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Project Goals: This project asks how ecological interactions (cooperative and antagonistic) within the soil microbiome influence soil carbon cycling and persistence. The researchers' primary goals are to (1) test how 23 years of climate change alter microbial interactions and affect the fate of soil carbon, (2) quantify microbiome interactions that change the biochemical community-scale efficiency of carbon use and its fate, and (3) infer ecological interactions using machine learning and ecological models.

Soil ecosystems are critical in the global carbon budget, and climate change can disrupt their functioning. In a northern Arizona climate change experiment, long-term alterations in temperature and precipitation have changed plant composition and primary production, leading to increased ecosystem respiration and photosynthesis but reduced soil carbon levels. However, much remains unknown about how microbial trophic interactions influence soil nutrient dynamics and how climate change affects these interactions. The research team hypothesizes that warming initially triggers cooperative interactions for complex carbon source degradation and top-down control of microbial communities by protists. Over time, as available carbon is depleted, predatory bacteria and viruses become the dominant top-down forces, altering predation modes and the fate and persistence of soil carbon.

To test the team's hypotheses, researchers are conducting parallel field and laboratory experiments. In the field, researchers added plant roots highly labeled with carbon-13 (^{13}C) to mixed conifer forest soils under warmed and unwarmed conditions in field mesocosms to trace carbon flow through the microbial food web.

Preliminary findings suggest that while total ecosystem respiration remained relatively constant in both conditions, plant root respiration was approximately 1.5 times higher in warmed soil than in unwarmed soil. Ongoing metatranscriptomics, metagenomics, and quantitive stable-isotope probing (qSIP) amplicon sequencing will identify potential trophic interactions driving carbon utilization dynamics.

In the laboratory, researchers are conducting trophic manipulation experiments using mixed conifer forest soils to investigate how predatory protists and bacteria influence carbon fate. Initially, microbial enrichments derived from researchers' field soils, including diverse populations across life domains, were established. Prevalent microbial communities in the enrichments, identified as high-quality metagenome-assembled genomes, include bacterial groups such as *Bacteriovorax* sp., *Pseudobdellovibrio* sp., *Bdellovibrio* sp., *Rhodoferax* sp., *Pedobacter* sp., and *Burkholderia* sp.; protists like *Spumella* sp. and *Acanthamoeba* sp.; and viruses such as *Mimivirus* sp. and *Kisquinquevirus* sp.

The reintroduction of enriched protists and their prevalence in soil microcosms, along with their impacts on bacterial communities, were assessed by quantifying gene expression using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of 18S rRNA and 16S rRNA. Expression of 18S rRNA genes in protist-treated soils (soil and protists in water to 60% water-holding capacity) was 90-fold higher than in controls (soil and water only), indicating a higher protist prevalence. Anticipated lower 16S rRNA expression in protist-treated soils suggests antagonistic associations. Ongoing oxygen-18 qSIP amplicon sequencing analysis will confirm findings and allow the calculation of the growth rates of targeted communities.

Predatory bacteria isolated from microbial enrichments will be used in trophic manipulation experiments alongside sorted protist populations to track ^{13}C's fate from labeled plant roots and study ecological dynamics and microbial community interactions.

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Microbial Networks Demonstrate Extraordinary Metabolic Versatility and the Ability to Obtain Electron Acceptors from Soil Organic Matter in Temperate Peatland Soils

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Project Goals: The goal of this project is to elucidate the fundamental principles driving physiology and metabolic exchange within microbial interaction networks that regulate the rate-limiting steps in soil organic matter (SOM) degradation, specifically the oxidation of phenolic compounds derived from lignocellulose and lignin-like polymers in carbon-rich peatlands and their roles in the preservation of organic matter under anaerobic, water-saturated conditions. The project combines multiomics with advanced analytical chemistry to test the "enzyme latch" hypothesis and its response to climate change drivers. Field and laboratory investigations will be integrated to construct and calibrate a predictive framework that links specific microbial processes and interactions to the mechanisms driving the rate-limiting steps of enzymatic SOM decomposition (i.e., phenolic compound oxidation and hydrolysis), SOM persistence, and greenhouse gas production in peatland soils. To investigate the response of microbial communities to climate change drivers, researchers leverage DOE's Spruce and Peatland Responses under Changing Environments (SPRUCE) experiment where air and peat warming are combined in a whole-ecosystem warming treatment.

Peatlands represent climate-critical regions that cover only 3% of the Earth's land surface but store approximately one-third of all soil carbon (C). The future role of peatlands in C sequestration remains uncertain and depends on the impact of global change–related perturbations on their C balance. In this project, researchers defined the microbial networks that regulate belowground C turnover by combining a genomic-centric metagenomics approach with biogeochemistry and metabolomics. The team analyzed 131 metagenomes (totaling 2.4 terabase pairs of sequences) obtained from soil samples collected to 2 meters depth in the peat column between 2015 and 2018, reconstructing 697 metagenome-assembled genomes (MAGs). Surprisingly, researchers found that only 2% of the MAGs retrieved from the SPRUCE site were shared with those identified in well-studied European peatlands where soils experience similar environmental conditions. Microbial community composition and functional potential are strongly depth-stratified and closely parallel changes in activity, redox, and organic matter quality. Overall, the metabolic pathways identified within the MAGs reveal a high-metabolic potential for sulfate/sulfite reduction, denitrification, methanogenesis, and homoacetogenesis implicating which of these are important terminal electron accepting processes. The dominant methanogens detected (*Methanoflorens*) demonstrate the potential to carry out acetoclastic as well as hydrogenotrophic methanogenesis, which has been only described previously for the genus *Methanosarcina*. In addition, the team uncovered a large diversity of sulfate/sulfite reducers and acetogens that were not previously associated with peatlands. The results indicate that C degradation is electron acceptor–limited and mediated by a much broader repertoire of anaerobic respiration processes than previously thought, likely supplied by electron acceptors derived from the soil organic matter itself. Despite the dramatic increase in gaseous emissions (e.g., carbon dioxide and methane) with warming over the same period, microbial diversity and composition remained stable, indicating slow growth and a resistant soil ecosystem. However, the genomic potential for methylotrophic methanogenesis was stimulated while homoacetogenesis was hampered by warming.

Reseachers took advantage of a generational drought that occurred in 2021 at the SPRUCE site to investigate the combined impacts of warming and drought on the belowground C cycle. The team hypothesized that the warmed, dried peatland will be released from the enzyme latch, thereby accelerating soil organic matter decomposition by enhancing the oxidation of phenolic compounds. During and postdrought, phenolic degradation and C-activated gene expression, as well as enzyme activity, increased, likely driven by heightened fungal activity.

Conversely, climate change–induced water-table drawdown reduced the activity of versatile polyphenol-degraders as well as the expression of anaerobic phenolic compound transforming genes. Temperature influenced the microbial community's recovery postdrought, with warmer treatments exhibiting gene-expression patterns more divergent from the predrought profile compared to ambient conditions. This research indicates that phenolic compound degradation is more complex than the enzyme latch suggests, emphasizing the need for a deeper understanding of microbial processes to accurately predict the impact of climate change on peatland C storage.

To determine whether warming-induced shifts in plant species composition may act to bolster the enzyme latch through the accumulation of plant-derived phenolic compounds that inhibit microbial SOM decomposition, the team conducted a seasonal study of soluble phenolic

compound concentrations across the SPRUCE temperature treatments. Phenolic compounds are highly sensitive to temperature and exhibit the greatest concentrations (by a factor of four) in the warmest treatments where shrubs, coincidentally, have significantly increased in biomass relative to other types of vegetation. Phenolic compounds, normalized to total dissolved organic matter (DOM) concentration, show a 50% increase across seasons in all plots. These data indicate that both phenolic compounds and DOM increase with growing season and temperature, but that phenolic compounds are either more recalcitrant over the annual cycle or they are produced and retained at a higher rate. In addition, researchers performed a comparison of peatland sites that vary in plant species composition, temperature, and pH (3.5 to 6.5) across a latitudinal gradient. The team observed a significant negative correlation between soil pH and soluble phenolics, with low-pH sites showing up to 5-fold higher phenolic concentrations. Researchers are currently quantifying decomposition rates in soils from all peatlands sampled to explore the controls of C turnover across peatland types.

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Understanding the Role of Permafrost-Affected Microbes in Thawing Arctic

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Project Goals: Using integrative metaomics technologies, determine the roles of natural microbial populations in pristine permafrost, seasonally thawed active layers, and hydrogeologically connected fjord sediments in the degradation of organic matter and their contributions to climate feedbacks in the thawing Arctic.

Permafrost and permafrost-affected areas cover approximately 24% of the global terrestrial surface and reserve ~50% of total soil organic carbon. Global warming drives permafrost degradation, release of organic carbon to decomposition, and intensification of microbial activity, which in turn increase greenhouse gas flux and exacerbate climate change feedbacks. Biodiversity of heterotrophic microbes and the metabolic pathways they use to convert newly available organic matter to carbon dioxide and methane are little studied. Increased availability of organic carbon from thawing permafrost threatens to create a positive feedback on climate change, and since thawing organic carbon is transported by subterranean groundwater flow into nearby rivers or fjords, these microbial feedbacks involve communities in thawing permafrost as well as those in hydrogeologically connected soils. Greenhouse gas emissions at the soil surface are an amalgamation of microbial activity in all the layers underneath, so knowing the vertical layering of microbial communities is key to understanding the mechanisms of these climate change feedbacks.

Svalbard, Norway (79°N), is experiencing faster warming than the rest of the high Arctic, making it a bellwether for Arctic permafrost. Samples of permafrost and active layer were collected during winter at the Bayelva field site, and fjord sediments were collected during spring in close proximity to Ny Ålesund, Svalbard. The team examined *in situ* microbial communities using metagenomics, culturing, and extracellular enzyme assays from the surface down to 141 cm. Researchers compared the vertical layering of these communities and their carbon-degrading genes to those found in the adjacent marine fjord sediments using metagenomics, metatranscriptomics, and metaproteomics. Using comparative metagenomics and metagenome-assembled genomes (MAGs), the team showed the depth distribution of individual MAGs leads to layered activity. The higher abundance of genes and peptides for major carbohydrate active enzymes (CAZymes) and glycoside hydrolases in subsurface at depths around 30 cm and 80 to 90 cm suggests that subsurface microbial communities are more active due to insulation from harsh surface conditions and high liquid-water content, even though the deeper soils are sustained by older deposits of organic matter. Importance of the phyla of Verrucomicrobia and Proteobacteria was shown in both fjord sediments and permafrost-affected soils. Even though some matches between organisms that are capable of degrading similar organic matter in soils and the fjords were shown, the activity profiles differed from taxonomic profiles based on genetic potential alone. Researchers found significant overlap in potential for organic matter degradation in the subsurface of both the permafrost active layer and the fjord. It is likely that these subsurface communities are supported by the higher liquid-water contents in the soil subsurface as well as depth-related changes in terminal electron–accepting

processes in fjord sediments. This suggests a direct role of subsurface microbial communities in a potential feedback loop with climate change, where thawing permafrost releases organic matter to active microbes, which, in turn, convert the organic matter to greenhouse gases that may further warm the climate.

Abuah, F., et al. "Investigating Microbial Communities in Svalbard Permafrost," *AGU Fall Meeting*. Chicago, Ill. 12–16 December 2022. B12I-1154.

Abuah, F., et al. "Subsurface Microbes May Drive Climate Feedbacks in Thawing Arctic Permafrost," *Geobiology GRC*. Galveston, TX. 14–19 January 2024, #33.

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The Complex System of Organic Carbon Remineralization in Rapidly Thawing Svalbard Permafrost and Active Layer Soils

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Project Goals: Researchers will determine the factors within a complex natural microbial community that dictate how much carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) are released in thawing permafrost, including the precise mechanisms of soil organic carbon (C) degradation by specific microbial community members in permafrost from Svalbard. This research focuses on a critical area of the Arctic, with high rates of storm intensification, temperature increase, and permafrost thaw. The results from this study will therefore be predictors for the future of permafrost thaw in the rest of the Arctic.

Arctic soil communities are on the frontline of the global response to climate change. This multi-institutional and multi-international collaboration has tackled the complexity of microbial organic matter remineralization across permafrost and active layers, as well as sediments from both of these locations that have been redeposited onto the fjord floor by seasonal glacial melt. Researchers completed two field seasons in Svalbard, Norway, despite the COVID-19 pandemic, a new war affecting the participation of some of the collaborators, and the passing of a member of the leadership team who was also a dear friend. Researchers have leveraged this project with international collaborators who have greatly expanded the breadth of the original project to mutually enhance this work. This projected is reporting: (1) a multiomics approach for examining *in situ* microbial communities combining metagenomics, metabolomics, metatranscriptomics, and metaproteomics with geochemical measurements to recreate depth gradients across the soils and fjord sediments; (2) incubation studies examining gas fluxes and uptake of labeled substrates into the active fraction of microbial biomass; (3) development of molecular dynamics models to understand the interactions of C-degrading enzymes with mineral surfaces; and (4) multiseasonal metabolic flux measurements to yield system-wide net process rates. Across all these different metrics, researchers find that the activity and diversity of microbial communities, as well as their abilities to break down different sources of organic matter and the amount of gases that result from that, changes with depth in unexpected ways. Most notably, the well-established steep drop in microbial activity with depth that is present in most temperate ecosystems does not occur in the Svalbard active layer soils or in fjord sediments. By partnering with a long-term monitoring station in Bayelva, researchers have seasonal variation measurements of temperature and liquid-water content from over 20 years, showing that soils buried tens of centimeters have higher volumetric water content due to insulation from surficial soils (Sipes et al. 2024). Fjord sediments, in turn, have access to a deep reach of oxidized sulfur and C compounds, stimulating a burst of transcriptional activity in a range of fermentative and respiratory microorganisms. The pathways and activities for C degradation differ greatly by depth in both systems, as well as by location across the permafrost and fjord system. This implies that the organic matter in freshly thawed permafrost may be immediately available to microbial degradation, since the work demonstrates that the deeply buried communities are already active due to the higher liquid water availability. The presence of a new set of degradational capabilities in the fjord sediments suggests that even if organic matter from freshly thawed permafrost is not degraded *in situ*, it has a secondary chance to be degraded after being swept into the fjords from glacial runoff. In total, a picture is emerging of the permafrost and fjord system as being a larger scale "factory" for processing

thawing permafrost, with the subsurface playing a key role, possibly amplifying the rate of CO_2 production beyond what occurs in surficial terrestrial soils alone.

Lloyd, K.G., et al. Submitted 2024. "Depth-specific distribution of acidobacterial classes in permafrost active layer in Ny Ålesund, Svalbard (79°N)."

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Converting Methoxy Groups on Lignin-Derived Aromatics from a Toxic Hurdle to a Useful Resource: A Systems-Driven Approach

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Methoxylated aromatics, originating from lignin hydrolysis, are toxic substrates for many species when consumed at higher concentrations. This is due to their aromaticity and the formaldehyde that is generated internally from the cleavage of methoxy groups. Researchers have discovered novel genetic factors in *Methylobacterium* involved in methoxylated aromatic metabolism and formaldehyde tolerance. The project has focused on multiple aspects: (1) characterizing the native pathway of vanillate (VA) catabolism in a natural strain of *Methylobacterium*; (2) expressing the VA pathway into a genetically tractable *Methylobacterium* strain and using experimental evolution to improve VA utilization; (3) characterizing the aromatic and formaldehyde stress response to VA; and (4) understanding the phenotypic heterogeneity of polyhydroxybutyrate (PHB) production using synthetic biology and novel single-cell microscopy. Results have shown that *Methylobacterium* is a robust system for the utilization of lignin hydrolysis byproducts and their conversion to value-added products.

Root-Mediated Impacts of Plant Volatile-Organic-Compound Emissions on Soil Carbon

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Project Goals: The overarching project goal is to verify and quantify volatile organic compounds (VOCs) as direct and indirect contributors to soil carbon (C) stabilization within the rhizosphere and beyond through teleconnections, and to determine their underpinning ecological and metabolic mechanisms. The long-term motivation for this project is to transform the current conceptual understanding and predictive capacity of microbial systems and soil C stabilization to include the important roles of volatile compounds. This falls under the group's objective to determine the contributions of root-released VOCs and VOC transformations by soil microbiomes to soil C cycling and stabilization. Specifically, in these tasks, researchers quantify subsurface root and soil VOC cycling to determine how deep soil warming influences soil C in a coniferous forest and how plant productivity, root biomass, and plant growth stages influence soil C in an agroecosystem.

Plants are recognized as the dominant source of biogenic VOCs to the atmosphere, where they play critical roles in air quality and climate, yet the parallel impact of plant-derived VOCs on the pedosphere (soil) remains poorly quantified. VOCs released by decomposing litter can contribute to soil C pools, including those associated with soil C stabilization, and researchers hypothesize that root VOCs can also contribute to these soil C pools. Furthermore, researchers anticipate this pathway for soil C stabilization will depend on plant physiological traits (e.g., photosynthesis, growth rates, and stomatal conductance), rhizosphere microbes and their activities, and soil environmental factors. Currently, rhizosphere VOC cycling remains poorly described, in part due to a lack of developed methods. In this project, the group integrated new *in situ* and nondestructive approaches for measuring root VOCs and tracking their fates in soil.

The group designed two separate rhizobox systems to measure VOCs from soil and rhizosphere from ponderosa pine seedlings and soil from the temperate coniferous Blodgett Experimental Forest in the Sierra Foothills in California. Both systems passively collected soil gas using diffusive teflon samplers shaped either as a cylindrical soil gas probe, as researchers have described previously (Roscioli et al. 2021; Gil-Loaiza et al. 2022), or a diffusive sheet connected to a large artificial macropore. Subsurface VOCs and carbon dioxide (CO_2) in soil (soil only) or rhizosphere (soil and

plant) treatments were measured using a suite of online gas analyzers including a proton-transfer-reaction mass spectrometer, a thermal-desorption gas-chromatography mass spectrometer, and a cavity ring-down spectroscope over a 6-day period with a diurnal light and temperature program (light 6:00–20:00, 6:00: 15°C, 14:00: 35°C). Alongside higher levels of CO_2 (rhizosphere respiration), the group found elevated soil gas concentrations of methanol, acetic acid, acetone, and acetaldehyde in the pine tree rhizosphere compared to soil alone, indicating a root source. Soil appears to have been a source of 1,2-butadiene and isoprene (fragment) that were elevated in both treatments. These results are consistent with the previous discovery of high concentrations of volatile compounds, including methanol at Blodgett (nuclear magnetic resonance on soil extracts), suggesting that pine roots may be an important source of these compounds and that the rhizobox systems are a useful tool for capturing and partitioning VOC sources and sinks in the rhizosphere. Ongoing research is comparing the performance of the two rhizobox systems and performing experiments to evaluate the impact of soil warming and moisture availability on root and soil VOC cycling and their contributions to soil carbon under controlled conditions. These results will be compared to those from the group's upcoming field campaign at the deep soil warming experiment in the Blodgett Research Forest.

Gil-Loaiza, J., et al. 2022. "Versatile Soil Gas Concentration and Isotope Monitoring: Optimization and Integration of Novel Soil Gas Probes with Online Trace Gas Detection," *Biogeosciences* **19**(1). DOI:10.5194/bg-19-165-2022.

Roscioli, J. R., et al. 2021. "Soil Gas Probes for Monitoring Trace Gas Messengers of Microbial Activity," *Scientific Reports* **11**(1). DOI:10.1038/s41598-021-86930-8.

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Transformations of Soil Organic Carbon Influenced by Volatile Organic Compounds

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Project Goals: Volatile organic compounds (VOCs) are ubiquitous carbon (C) pools in the Earth system, but often remain uncharacterized as vectors of soil organic C (SOC) transformations. Roots, litter, aboveground vegetation, and microbial metabolism are all sources of VOCs. However, little is known about how these omnipresent metabolites can contribute to C cycling in soils. This project aims to verify and quantify the direct contributions of VOCs to soil C pools and determine their underpinning ecological and metabolic mechanisms. Moreover, it aims to understand how VOCs connect distant metabolic and biochemical regions through their high mobility in soil. The long-term motivation for this project is to transform the current conceptual understanding and predictive capacity of microbial systems and soil C stabilization to include the important roles of volatile compounds.

Volatile organic compounds (VOCs) are diverse and prevalent metabolites exchanged in microbial systems but are often overlooked as vectors of SOC transformations (Honeker et al. 2021; Meredith et al. 2022; Meredith et al. 2023). Roots, litter, aboveground vegetation, and microbial metabolism (Honeker et al. 2023) are all sources of VOCs to soil; however, little is known about how they can contribute to soil C cycling. Microbial uptake of VOCs by soil is increasingly recognized as a ubiquitous process, largely unconstrained by observations. VOCs can contribute to key soil C pools, including microbial biomass, dissolved organic matter, particulate organic matter, and mineral-associated organic matter (MAOM), suggesting that they can participate in critical soil C stabilization pathways such as the microbial necromass conduits to MAOM. Yet, understanding is still lacking regarding the fate of VOCs entering the soil system and the specific VOC-induced transformations they may elicit in SOC, hindering the characterization of this process across soil and volatile compounds. To address this research gap, the research team designed complementary studies to evaluate (1) the fate of VOCs added to soil

and (2) the contributions of these VOCs to SOC pools in soil from a semi-arid agroecosystem.

In the first experiment, commonly observed VOCs were added into the subsurface of soil columns (100 cm depth) and the concentrations of added VOCs and their gas-phase degradation products were monitored using subsurface gas sampling probes (Roscioli et al. 2021; Gil-Loaiza et al. 2022) at different distances (7 points) from the source. Results indicated that all VOCs were consumed by soil, with the net consumption rates of many increasing over time, indicating microbial acclimation to increased substrate availability or sorption interactions. Interestingly, certain VOCs exhibited greater mobility in the soil compared to others, evidenced by their abilities to diffuse over longer distances. This discrepancy in mobility highlights the diverse potential of VOCs to influence SOC levels in adjacent regions, potentially establishing VOC teleconnections within the soil environment.

Finally, partially oxidized volatile products of microbial VOC consumption pathways were observed, revealing the presence of microbes capable of oxidizing isopropanol and acetone.

The second experiment involved a soil incubation study to evaluate the contributions of VOCs to SOC pools using a subset of the compounds tested above. The research team evaluated whether the diversity and quality of SOC changed in response to weekly additions of five individual VOCs over a 3-month period: methanol, acetone, acetaldehyde, isoprene, and α-pinene. Carbon dioxide concentrations were monitored regularly as a proxy for microbial activity. High-resolution SOC analysis by Fourier-transform ion cyclotron resonance mass spectrometry (FTICRMS) revealed that the different VOCs facilitated unique SOC transformations through microbial, as well as potentially abiotic, processes. Specifically, pinene, methanol, and acetaldehyde drove changes in lipid-like compounds, which represent SOM composition, possibly due to microbial biomass or metabolic pathway activation. External VOC exposure is presumed to have had a priming effect that trained the indigenous microbes to assimilate subsequent VOCs. This study aims to grow understanding of the role of VOCs in soil C cycling and their contributions to soil ecological and metabolic interactions related to C stabilization.

Gil-Loaiza, J., et al. 2022. "Versatile Soil Gas Concentration and Isotope Monitoring: Optimization and Integration of Novel Soil Gas Probes with Online Trace Gas Detection," *Biogeosciences* **19**(1), 165–85.

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Meredith, L. K., and M. M. Tfaily. 2022. "Capturing the Microbial Volatilome: an Often Overlooked 'Ome'," *Trends in Microbiology* **30**, (7), 622–31.

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Roscioli, J. R., et al. 2021. "Soil Gas Probes for Monitoring Trace Gas Messengers of Microbial Activity," *Scientific Reports* **11**(1), 8327.

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Characterizing Bacterial–Fungal Interactions Within Soil Niches and Across Soil Mineralogies

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Project Goals: This work aims to develop a quantitative and mechanistic framework for understanding how bacterial–fungal interactions (BFIs) influence carbon (C) stabilization and mineralization within soil niches and across soil mineralogies. Leveraging principles of community systems biology and ecology, this experimental strategy combines stable isotope probing (SIP), SIP-assisted metaomics, field mesocosms, soil process rate monitoring, and microbe-informed ecosystem modeling. Objectives include: (1) investigating the influence of various C sources (e.g., rhizodeposits, hyphal deposits, and litter) on grassland BFIs and their subsequent effects on the fates of these photosynthates;

(2) examining the role of BFIs in promoting C stability within soil aggregates and on mineral surfaces, and their impacts on C destabilization in soils across mineralogies; and (3) assessing how drought conditions, in conjunction with C source and soil mineralogy, shape BFIs and the soil processes they govern.

Bacteria and fungi are dominant soil microbes that play crucial roles in biogeochemical cycling. While cross-domain interactions in soil are well-documented, a mechanistic understanding of BFIs and their influences on biogeochemical cycling of essential soil nutrients under differing soil mineralogy is still lacking. This study comprises a field experiment at the University of California Hopland Research and Extension Center with ingrowth cores of different mesh sizes to separate soil niches. These cores were incubated in a randomized block design plot under rain-out shelters and subjected to either 90% or 50% of ambient precipitation. A subset of hyphosphere cores (i.e., 44μm mesh allowing fungal hyphae and bacteria to permeate but excluding roots) and negative controls (i.e., 0.45μm mesh preventing hyphae from crossing) were excluded from receiving rhizodeposits and litter for two growth seasons. These treatments represented C-depleted conditions. The research team monitored carbon dioxide (CO_2) efflux from the cores and soil moisture levels within the plots. Preliminary findings from a mixed-effects model indicated a significant effect of both precipitation level and core type on CO_2 efflux. Efflux was, on average, 10% lower under 50% precipitation from September 2023 to January 2024. This was consistent with 14% lower soil moisture under 50% precipitation from October to December 2023.

Furthermore, a significant interaction between mesh size and soil moisture was observed to influence CO_2 flux rates. Efflux from the 44μm mesh cores was 15% higher than that from the 0.45μm cores, likely linked to fungal hyphae activity within the 44μm mesh cores. Also being monitored is CO_2 efflux from 830μm mesh cores representing the rhizosphere (i.e., allowing roots, fungal hyphae, and bacteria to cross) to gain further insights into the role of BFIs on CO_2 flux dynamics across soil niches. In spring 2024, the research team aims to label the grasses growing adjacent to the cores with $^{13}CO_2$ followed by soil sampling for chemical and microbial analyses. The objective is to quantify the transportation of photosynthetic C into the cores via hyphae, identify the soil C pools that the photosynthetic C is transformed into, and characterize the bacterial and fungal taxa involved in these processes.

To characterize BFIs across soil mineralogy, this project aims to conduct a parallel field-based mesocosm experiment in which the same ingrowth cores will be deployed into intact megaliths of five soil types with distinct clay mineralogies from Hawai'i's O'ahu Island. A similar $^{13}CO_2$ labeling event will be carried out to measure how soil mineralogy interplays with BFI-mediated C dynamics.

Lastly, the research team tested model frameworks to represent the diversified interactions between bacteria and fungi in soils. Sensitivity analysis demonstrated that the initial fungi-to-bacteria ratio and fungi/bacteria enzyme production rates are key parameters regulating competition between bacteria and fungi. After developing a suitable model framework, the team aims to integrate CO_2 effluxes, C pool sizes, ^{13}C enrichment, and SIP-derived metagenomic data from the above experiments into a new generation of omics-informed, niche-identified Microbial ENzyme Decomposition model.

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Flow Sorting and Sequencing Active Environmental Viruses from Methane-Oxidizing Communities with Viral-BONCAT

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Project Goals: The goals of this project are to (1) develop a mechanistic understanding of anaerobic oxidation of methane (AOM) syntrophic interactions; (2) define and functionally characterize the microbial community, including viruses, associated with methanotrophic consortia under changing environmental conditions; and (3) create an integrative modeling framework to explore the ecophysiology of AOM consortia and their community interactions in environmental context.

Beyond identifying the microorganisms present in the environment, characterizing their interactions and impacts on other biological communities is becoming increasingly necessary to understand the functioning of ecosystems. One of the major regulators of biological communities are viruses, capable of infecting and killing a broad range of organisms across the tree of life. In recent years, metagenomics has significantly expanded the knowledge about the virosphere and its

diversity (Laso-Pérez et al. 2023). In this project, researchers present the development of viral bioorthogonal noncanonical amino acid tagging (BONCAT)–fluorescence-activated cell sorting (FACS) coupled with metaviromic sequencing, where free viruses that have recently infected an active cell are specifically labeled with BONCAT and sequenced from complex environmental communities. This newly developed approach for sorting, quantifying, and sequencing active viral-like particles offers a new lens in which to track viral host dynamics and to characterize the selective pressure of distinct viral populations on microbial communities within diverse ecosystems.

Viral BONCAT-FACS is based on the BONCAT methodology. Its new application to the virosphere was demonstrated in a laboratory at Caltech, where free viruses were visualized using epifluorescence microscopy after incorporating newly synthesized peptides or amino acids from their active hosts (Pasulka et al. 2018). This optimization of viral BONCAT includes an enhancement in the fluorescence signal associated with active viruses, enabling detection by flow cytometry. Viral BONCAT-FACS, like the previously developed high-throughput FACS (HT-FACS) in the Martinez-Garcia group enables genomic analysis of flow-sorted viral populations, allowing the differentiation of active, newly produced BONCAT-labeled viruses, and the nonactive viral particles concurrently with active and inactive cell fractions (Martinez-Hernandez et al. 2017). The team successfully amplified and sequenced complete genomes using the WGA-X reaction, initially demonstrating the viability of this technique by sequencing more than 1,000 contigs from the active viral fraction in coastal waters, followed by further optimization for sediment- and rock-associated microbial communities catalyzing the AOM (Stepanauskas et al. 2017; Garcia-Heredia et al. 2021).

Viral BONCAT-FACS was used on laboratory-maintained methane-fed sediment and AOM microbial mat incubations. After a 3-day BONCAT incubation in the presence of methane (CH_4), active viruses and microorganisms were fluorescently labeled with the optimized click reaction and sorted, yielding ~3,500 active viral-like particles (VLPs) and ~500 active microbial cells. Sequencing and bioinformatic analysis of the amplified genomes from both viral fractions confirmed the recovery of diverse viral genomes. In a second BONCAT experiment, AOM mat samples enriched in anaerobic methanotrophic (ANME) archaea and sulfate-reducing bacteria were incubated with either methane (CH_4) and SO^{-2} or CH_4 and AQDS (e.g., decoupling ANME archaea from their sulfate-reducing partners; Scheller et al. 2016). After 4 weeks of incubation, higher cellular and viral BONCAT activity was observed in the AQDS treatment. Genomes of 75,000 active and 75,000 nonactive VLPs, along with more than 100,000 active and nonactive microbial cells were sorted and sequenced from the different treatment conditions. Sequencing is now underway and will help illuminate how viruses regulate the dynamics of these methane-fueled communities and how viral pressure is affected by cellular stress conditions.

Garcia-Heredia, I., et al. 2021. "Benchmarking of Single-Virus Genomics: A New Tool For Uncovering the Virosphere," *Environmental Microbiology* **23**(3), 1584–93. DOI:10.1111/1462-2920.15375.

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The Enigma of N_2 Fixation in Energy-Limited Anaerobic Methane-Oxidizing Microbial Consortia

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Project Goals: The goals of this project are to (1) develop a mechanistic understanding of the anaerobic oxidation of methane (AOM) syntrophic interactions; (2) define and functionally characterize the microbial community, including viruses, associated with methanotrophic consortia under

changing environmental conditions; and (3) create an integrative modeling framework to explore the ecophysiology of AOM consortia and their community interactions in an environmental context.

Most elemental cycles in Earth's surface environment are mediated by microbial reactions. To quantify these transformations and predict the distribution of nutrients and other chemicals of interest, biogeochemical models need to capture the transport and reaction rates of these processes, which depend on environmental conditions such as the availability of substrates, metabolic pathway expression, and physiological constraints.

In this project, researchers explore environment–microbe interactions through reactive transport modeling of the microbially mediated AOM. First, researchers show simulations of spatially resolved microbial consortia composed of anaerobic methanotrophic (ANME) archaea coupled metabolically to sulfate-reducing bacteria via direct interspecies electron transfer (He et al. 2021). Growth efficiencies derived from estimates of catabolic energy yields, anabolic energy requirements, and energy dissipation (Heijnen and Van Dijken 1992) resulted in growth yields consistent with observations when using ammonium as the nitrogen (N) source.

The team's model simulations showed that N demands can likely be fulfilled without causing significant ammonium drawdown within or surrounding the microbial aggregates. Nevertheless, some archaea and bacteria involved in AOM, a process with limited energy yield, have been shown to fix N_2 (Dekas et al. 2018; Metcalfe et al. 2021), which requires a significant amount of adenosine triphosphate and reducing equivalents. When extending this model to allow for N_2 as the N source, the predicted growth yields decreased but remained substantially higher than yields derived from measurements when N_2 fixation was active, suggesting that physiological controls are important.

To further investigate possible triggers for this energy-consuming process, researcherse studied growth and its dependence on N processing using a flux-balance model of ANME (Faria et al. 2023). The simulations showed that even significant leakage of N-rich compounds is unlikely to induce N_2 fixation. Researchers therefore explored the potential of the use of N_2 fixation to maintain intracellular redox homeostasis as has recently been proposed for *Geobacter sulfurreducens* (Ortiz-Medina et al. 2023). However, researchers were unsuccessful at inducing N_2 fixation in the model under environmentally relevant conditions, which points to as-yet poorly understood features of this energy-limited syntrophic partnership and the need for additional studies of the metabolic controls in ANME archaea.

Finally, environmental conditions within sediments, soils, and rock matrices may also vary on small spatial scales, depending on the pore connectivity, which could lead to conditions that trigger different metabolic activities. To explore the potential for the formation of distinct microenvironments within carbonate rocks that are formed through the process of sulfate-coupled anaerobic methane oxidation, the team developed a Lattice-Boltzmann porescale reactive transport model (CompLab3D). In these simulations, researchers established the model domain from CT scans of carbonate rocks, and then quantified the connectivity of their pore spaces by computing the distribution of water ages. This distinguishes well-connected regions from isolated pores, which may support different microbiological processes and levels of activity within the carbonate structure.

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Systems-Level Insights into the Physiology of Methane-Fueled Syntrophy Between Anaerobic Methanotrophic Archaea and Sulfate-Reducing Bacteria

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Project Goals: (1) Develop a mechanistic understanding of anaerobic oxidation of methane (AOM) syntrophic interactions; (2) define and functionally characterize the microbial community, including viruses, associated with methanotrophic consortia under changing environmental conditions; and (3) create an integrative modeling framework to explore the ecophysiology of AOM consortia and their community interactions in an environmental context.

AOM is a geologically important process that impacts methane utilization in marine sediments. AOM is mediated by anaerobic methanotrophic archaea (ANME) and is made energetically feasible by coupling methane oxidation with the reduction of electron acceptors such as nitrate, metals, or sulfate. In sulfate-coupled AOM, ANME are found in an obligate syntrophic partnership with sulfate reducing bacteria (SRB). This syntrophic association is driven by direct interspecies electron transfer between partners colocalized in a multicellular consortium.

Using comparative phylogenomics, the research team and others have shown that ANME are polyphyletic, evolving multiple times from methanogenic ancestors (Evans et al. 2019; Chadwick et al. 2022). Analysis of gene phylogenies, locus organization, and sequence alignments suggests that during this process, each ANME clade has convergently evolved to encode distinctive genes often involved in central metabolic pathways. In particular, studies of the most recently evolved ANME-3 clade have produced a step-by-step view of this process. Results suggests that evolution of convergent modifications to proteins involved in carbon and energy metabolism precedes later optimization through horizontal acquisition of multi-heme cytochromes and genes involved in nutrient acquisition and cell–cell interactions. Sulfate-reducing syntrophic bacterial partners have also convergently evolved from free-living sulfate reducers to syntrophic SRB by adapting their energy metabolism and acquiring genes by lateral gene transfer that promote interspecies interaction and biofilm formation (Murali et al. 2023).

In this work, environmental metaproteomics and metabolic modeling were used to test phylogenomics-derived inferences. Model results highlight differences in electron transport pathways that are critical to the differences between ANME and methanogens. Metaproteomics analyses of environmental ANME–SRB consortia demonstrated that previously identified pathways associated with ANME and SRB energy metabolism (e.g., Mcr, Dsr, Apr, and Rnf) and interspecies interactions (e.g., eCIS and adhesins) are actively expressed.

Additionally, the research team identified several highly expressed proteins that currently have no characterized function, highlighting additional unexplored aspects of AOM physiology. Targets were identified from highly expressed proteins for further heterologous expression (e.g., putative fibronectin binding matrix proteins, Rnf).

Further physiological insights of these slow-growing microbes will be gained by stable isotope probing metaproteomics (e.g., 13C-CH_4, 13C-$NaHCO_3$, 15N-NH_4Cl). Preliminary analysis shows uptake of labeled substrates by ANME–SRB, and upcoming analysis will provide insight into protein turnover, growth rates, and carbon uptake.

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From Viromes to Virocells: Dissecting Viral Roles in Terrestrial Microbiomes and Nutrient Cycling

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Project Goals: Develop paradigms for understanding the role of viruses and mobile genetic elements in soil ecology via ecogenomic inference and experimental interrogation of new soil-derived model systems, and to build tools (e.g., scalable new methods, new databases, and new model systems) to test these paradigms.

The activity of soil microbes affects global energy and nutrient cycles, but they do so under largely unconstrained but likely significant virus impacts. While viruses play pivotal roles in other ecosystems like the oceans, soil virus research is hindered by technical challenges. In this project, called VirSoil, the group focused on three aims: (1) detect, identify, and classify soil viruses and their potential roles; (2) mechanistically understand soil virus infections; and (3) develop community resources for studying soil viruses.

In Aim 1, the team elucidated soil virus ecology in a decadal bulk metagenomic dataset from a permafrost thaw gradient at Stordalen Mire. For DNA viruses, 5,051 virus populations were cataloged, documented to have high year-to-year turnover, and linked to carbon cycling through host prediction and gene content analysis that identified virus-encoded carbon degradation, methanotrophy, and methanogenesis genes (Sun and Pratama et al. In review.). For RNA viruses, nearly 9,000 sequences were identified, representing 2,651 novel "species," and then ecologically contextualized according to habitat, depth, and soil properties (Pratama and Dominguez-Huerta et al. In preparation.).

In Aim 2, the team sought to begin understanding soil virocells (i.e., virus-infected cells) by advancing virocell-specific analytics and developing new soil virus–host model systems. Towards this, researchers applied time-resolved, multiomics technologies to diverse virus genomes, infection efficiencies, and nutrient conditions to establish knowledge and protocol pillars using an established *Cellulophaga* virocell model system. This revealed that virus genome type and infection efficiency strongly shape bacterial biomolecule composition and dynamics, where virocell biomolecule specificity was highest in transcripts, lower in proteins, and lowest in metabolites (Howard-Varona et al. In preparation.) and post-translational modifications were uncovered (Peters et al. In preparation.). In parallel, multiomics of nutrient-limited *Pseudoalteromonas* virocells revealed the interplay between environment and virus infection intracellularly versus extracellularly (Howard-Varona and Lindback et al. In revision.). Finally, this aim also established random barcode transposon-site sequencing (RB-TnSeq) and CRISPR-Cas9 engineering approaches to assess virus components of the virus–host arms race and scalably characterize resistance mechanisms.

In Aim 3, the team focused on community empowerment. To this end, researchers established standard operating procedures for auxiliary metabolic gene analysis (Pratama et al. 2021), developed MetaPop to simplify population genetics analysis (Gregory et al. 2022), created an enhanced protocol for identifying and annotating metabolites through machine learning (Rajakaruna et al. In preparation.), curated an efam virus protein cluster database to improve virus protein annotation (Zayed et al. 2021), and worked with the DOE Systems Biology Knowledgebase to layer in basic iVirus functionality including virus identification (VirSorter and VirSorter2) and taxonomic classification (vConTACT2) tools. Finally, towards expanding model systems for soil viral ecology, the group screened hundreds of microbial strains to isolate viruses, triply plaque-purified subsets of these, and developed 60 viruses that were genome-sequenced and host-range-characterized as new virus–host model systems for virocell multiomics and other characterization (Gittrich et al. In preparation.).

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Exploring the Metabolic Capability of Undomesticated Thermophilic *Bacillus coagulans* for Biosynthesis of Designer Esters at Elevated Temperatures

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Project Goals: To fundamentally understand and redirect metabolism and regulation of thermophilic *Bacillus coagulans* for the efficient conversion of undetoxified lignocellulosic biomass hydrolysates into designer bioesters.

B. coagulans (now recognized as *Heyndrickxia coagulans*), a gram-positive facultative thermophile, thrives across a broad temperature range, utilizes undetoxified biomass hydrolysates, and synthesizes valuable chemicals like acetoin, butanediol, and lactate. This project exploits the robustness of *B. coagulans* for converting biomass hydrolysates into designer bioesters—such as acetate and lactate esters—widely used in fragrances, flavors, pharmaceuticals, and advanced biofuels. Through comprehensive screening of a library of diverse *B. coagulans* strains for desirable traits, researchers identified *B. coagulans* B-768 as an optimal host for metabolic engineering, given its capability to ferment C5 through C12 sugars, withstand undetoxified biomass hydrolysates, and produce significant lactate levels. Genome and proteome analyses highlighted B-768's expanded genome, coding for enhanced sugar utilization and lactate synthesis. Successful DNA transformation in B-768 has led to the creation of production strains harboring the exchangeable ester production modules to produce acetate and lactate esters at elevated temperatures, facilitated by engineered thermostable alcohol acetyltransferases. Notably, the team uncovered *B. coagulans* strains capable of producing valerate esters. Current efforts focus on elucidating *B. coagulans'* robust metabolism for complex hydrolysate utilization, improving synthetic biology tools (including transformation efficiency, plasmid stability, genome integration, and promoter and ribosome binding site optimization), and enabling modular cell engineering for selective biosynthesis of designer esters. Overall, *B. coagulans* is a promising microbial manufacturing platform that will be advanced by a fundamental understanding of its robustness, genetic engineering tool development, and the ability to harness it for production of designer bioesters from lignocellulosic hydrolysates.

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Response of Methanotroph Communities to Warming Temperatures in a Recently Thawed Fen

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Project Goals: To resolve key unknowns in the grand challenge of understanding the fate of carbon (C) in thawing permafrost, researchers focus on C-cycle climate feedbacks to warming in high methane (CH_4)-emitting landscapes in

an Arctic mire ecosystem. The team's aims are to (1) identify and resolve key gaps in the understanding of microbial C processes consequential to C storage and CH_4 emission, (2) identify and resolve a mystery of microbial oxidation rates and controls consequential to emission mitigation, and (3) integrate resolved consequential unknowns into next-generation ecosystem models.

As the climate warms, permafrost thaw is fueling high CH_4 emissions, particularly in permafrost peatlands. Aerobic methane-oxidizing bacteria (i.e., methanotrophs) could dampen these emissions, but how these microbes will respond to rising temperatures remains unknown. Researchers conducted laboratory incubations to investigate how methanotroph communities respond to warming temperatures at different peat depths in a recently thawed fen in a thawing permafrost peatland located in Stordalen Mire, Sweden. The team used 16S rRNA to characterize microbial community composition at 20°C and 25°C. Oxidation rates did not differ across peat depths (10-cm increments from 0 to 40 cm). Isotopic analysis of ^{13}C-CH_4 and ^{13}C-CO_2 will reveal sources of CH_4. Future work will investigate the metabolomic and genomic controls on CH_4 oxidation. These results will inform a trait-based ecosystem model to improve emission predictions under climate change.

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Influence of Temperature on Arctic Lake Sediment Methane Production and Organic Matter Composition

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Arctic lakes are important sources of methane (CH_4) into the atmosphere. CH_4 emissions from lakes are expected to increase as the Arctic warms due to increasing microbial activity and greater availability of more labile organic matter substrates. However, the effects of temperature on the sensitivity of different CH_4 production pathways and on the chemical composition of lake sediments remains understudied. In this project, researchers used incubations to measure the temperature sensitivity of sediment CH_4 production and sediment organic matter composition and diversity. CH_4 production increased exponentially with temperature across sediments from the edge and center of two lakes. Stable carbon (C) isotopic signatures of CH_4 and carbon dioxide suggest evidence of greater contribution of acetoclastic methanogenesis as opposed to hydrogenotrophic methanogenesis with increasing temperature, but the influence of anaerobic CH_4 oxidation on observed isotope signatures cannot be ruled out. CH_4 production was positively correlated with organic compounds that contained C, hydrogen, oxygen, nitrogen, and sulfur elemental compounds ($r^2 = 0.32$, $P < 0.01$). Further, the functional diversity (Rao's quadratic entropy) of elemental composition of sediment porewater was negatively correlated with activation energy derived from incubations, suggesting less elementally diverse sediments are more sensitive to temperature changes, despite the more elementally diverse sediments having higher production rates overall. The preliminary results highlight the complex interactions between organic matter diversity and CH_4 cycling. Pending microbial data (metaG and metaT analysis) will provide greater insights.

Rendering the Metabolic Wiring Powering Wetland Soil Methane Production

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Project Goals: Despite their vital roles in transforming nutrients and controlling greenhouse gas fluxes in wetlands, microbial knowledge is often limited to taxonomic identity alone and rarely includes cross-site comparisons. The team proposed to address this knowledge gap using

coordinated, reproducible field measurements collected across a wetland–methane continuum spanning geographical locations. This project tests the overarching hypothesis that microbial genomic attributes are conserved across high methane–emitting wetlands. First, researchers will use a cross-wetland approach to define the microbial membership, physiology, and interactions directly contributing to wetland methane production. Next, the team will uncover the microbial decomposition network features that classify high methane–emitting wetlands. Using this information, researchers will test the genomic resolution needed to make robust predictions of regional and global methane fluxes. These integrated field, laboratory, and modeling approaches will identify the unifying microbial properties governing soil carbon decomposition and methane fluxes, such that some level of biological representation in models will enhance predictions of soil methane fluxes.

To illuminate the metabolic features of carbon decomposition in high methane–emitting wetlands, the project created the Multiomics for Understanding Climate Change (MUCC) database. This resource contains the identity, distribution, and functional information of 26,000 microbial genomes from wetlands, including over 500 methanogen and methanotroph genomes. The team coupled MUCC to 133 spatially and temporally resolved metatranscriptomes, along with paired amplicon, geochemical, and greenhouse gas data from over 700 samples collected from one of the most prolific methane-emitting wetlands in the United States. From this site, researchers unveiled previously unrecognized roles of archaea in carbon decomposition, resistance to redox changes exhibited by methane-cycling community members, and the delineation of metabolic networks contributing to methane production. This standardized sampling and sequencing design set the stage for future cross-site comparisons, enhancing the ability to generalize findings across wetlands.

Extending the sampling from a single wetland, coordinated field sampling in year 1 added paired greenhouse gas measurements, soil chemistry, and multiomics from 12 additional wetlands (three bogs, four fens, and five marshes). Cross-wetland analysis of initial findings showed that in contrast to current paradigms, where acetolactic and hydrogenotrophic are the predominant forms of methanogenesis, methylotrophic methanogenesis is prevalent and active. Across sites, genome-resolved metatranscriptomes showed that members of the Methanomassiliicoccales, Methanosarcinales, Methanomethyliales, and Methanobacteriales were expressing methylotrophic genes, while metabolomics revealed new methylotrophic substrates (e.g., syringate and trimethyllysine). Further corroborating these findings, the team established enrichments of wetland soils dosed with either carbon-13 (^{13}C), dimethylsulfide, or ^{13}C acetate to demonstrate that field-relevant methylotrophic lineages were enriched in reactor ^{13}C-labeled proteomes. Likewise, methylated substrates considerably contributed to methane production (on average 53%), demonstrating that methylotrophic methanogenesis is a likely contributor to methane across global freshwater wetlands and should be included in process-based models.

To integrate this new microbial knowledge into ecosystem scale models, researchers built genome-scale metabolic models (GEMs) from MUCC genomes. MUCC GEMs combine field-derived multiomics data, enrichment-derived isotopic evidence, reference genomes, and fundamental physical and thermodynamic principles to produce the most accurate metabolic representation of each strain observed in the MUCC samples. These GEMs utilize a novel probabilistic framework that captures the metabolic diversity of each metagenome-assembled genome (MAG) by consolidating phylogenetically close references into a single GEM for simulation, allowing for the preferential simulation of common, conserved pathways while permitting niche pathways if conditions and constraints require it.

GEMs undergo initial testing for internal consistency, aligning with available experimental data. Then GEMs are merged into sample-level community models where GEMs are further refined to maximally recapitulate experimental data. This process tailors each sample to have a unique MAG model for each strain, whose metabolic behavior is described as how each strain transforms nutrients into biomass and byproducts. These whole-cell reactions, constrained by relative abundance from multiomics data, will ultimately be integrated into ecosystem-scale simulations. MUCC and the corresponding GEMs are publicly available on the DOE Systems Biology Knowledgebase (KBase), engendering collaborative enterprises with the goal to advance wetland climate-driven research. Ultimately, this research illuminates the metabolisms influencing the methane cycle, offering a direction for increased realism in predictive models of greenhouse gas emissions.

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Unraveling Metabolic Interactions Within a Rhizosphere Microbial Community

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Project Goals: This project couples novel laboratory and field studies to develop the first predictive model of grass microbiomes based on new mechanistic insights into dynamic plant–microbe interactions in the grasses *Sorghum bicolor* and *Brachypodium distachyon* that improve plant nitrogen (N)-use efficiency (NUE). The results will be used to predict plant mutants and microbial amendments, which improve low-input biomass production for validation in laboratory and field studies. To achieve this goal, researchers will determine the mechanistic basis of dynamic exudate exchange in the grass rhizosphere with a specific focus on the identification of plant transporters and proteins that regulate root-exudate composition, and how specific exudates select for beneficial microbes that increase plant biomass and NUE. Researchers will further develop a predictive plant–microbe model for advancing sustainable bioenergy crops and will predictively shift plant–microbe interactions to enhance plant biomass production and N acquisition from varied N forms.

This research delves into the rich microbial diversity present in soils, particularly in the rhizosphere, where a myriad of bacteria influences soil properties through nutrient transformations, including carbon and N pools that are directly linked to plant growth. To unravel the intricate web of metabolite exchanges among soil microbes and their dynamic interactions with the host plant, the team adopted a computational approach and utilized multiomics data (Kumar et al. 2019). Specifically, researchers focused on a synthetic microbial community (SynCom) composed of 16 rhizosphere bacteria isolated from switchgrass (Coker et al. 2022).

First, researchers constructed and manually curated genome-scale metabolic models for each of these rhizosphere bacteria, representing from various genera such as *Arthrobacter*, *Bacillus*, *Bosea*, *Bradyrhizobium*, *Brevibacillus*, *Burkholderia*, *Chitinophaga*, *Lysobacter*, *Methylobacterium*, *Mucilaginibacter*, *Mycobacterium*, *Niastella*, *Paenibacillus*, *Rhizobium*, *Rhodococcus*, *Sphingomonas*, and *Variovorax*. Integrating individual models with metatranscriptomic (RNA-Seq) and metatranslatomic (Ribo-Seq) data, researchers constructed condition-specific community metabolic models (CM-models). Throughout this investigation, the team systematically evaluated the impacts of removing individual microbes from the SynCom, shedding light on the specific contributions of each member. These CM-models predict the response of the SynCom to perturbation with very high accuracy. Furthermore, the CM-models played a crucial role in predicting metabolic exchanges between community members, unveiling the intricate nature of interactions, including competition and cooperation, among rhizosphere microbes.

These models have predicted substantial interactions involving the exchange of short-chain organic acids, carbohydrates, amino acids, and purine and pyrimidine derivatives among rhizosphere bacteria. Furthermore, the predictions suggest shifts in the nature of these metabolic exchanges when specific community members are removed. These model-driven hypotheses propose that such metabolic shifts confer nutritional advantages to select members, while concurrently suppressing the growth of others. Illuminating the intricate mechanisms of interaction among plant-associated microorganisms offers invaluable insights into the development of strategies for engineering microbial communities capable of enhancing plant growth and bolstering resilience against diseases.

Kumar, M., et al. 2019. "Modelling Approaches for Studying the Microbiome," *Nature Microbiology* **4**, 1253–67.

Coker, J., et al. 2022. "A Reproducible and Tunable Synthetic Soil Microbial Community Provides New Insights into Microbial Ecology," *mSystems* **7**.

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Science Focus Area
Bacterial–Fungal Interactions and Their Role in Soil Functioning

The Fungal Microbiome: Discovering and Investigating Novel Endohyphal Inhabitants

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https://genomicscience.energy.gov/lanlbfi/

https://sfa-bfi.edgebioinformatics.org/about

Project Goals: The Bacterial–Fungal Interactions (BFI) Science Focus Area (SFA) is focused on elucidating the mechanisms and implications of BFI on larger ecosystem functioning. Part of this work includes characterizing the interactions that occur within and immediately surrounding fungal hyphae (the fungal microbiome) between these fungal hosts, bacterial partners, and several other microorganisms.

Fungi are important members of larger, diverse microbiomes such as those in human guts, plant rhizospheres, or soil microbiomes. However, it has been demonstrated that fungi themselves can harbor complex microbiomes both within and directly surrounding the outer surfaces of their hyphae (Robinson et al. 2021). Endobacterial associates have been observed in both higher and basal lineages of fungi, but major questions remain regarding how these bacterial symbionts are acquired, how they persist within a fungal host, and how they impact fungal evolution and behavior. Using model interactions between Mollicutes-related endobacteria (MRE) and members of the Mortierellaceae, the group has discovered new insights into interaction persistence, potential coevolution, physical properties (e.g., distribution of MRE throughout hyphae), and functional impacts of MRE on fungal hosts (Longley et al. 2023). This work has led to the acceptance of a DOE Joint Genome Institute (JGI) New Investigator Community Science Program (CSP) proposal that will investigate the impacts of warming conditions on interactions between endobacteria and Mucoromycota taxa. This work will provide new insights into the mechanisms governing fungal–endobacterial interactions and will continue to help elucidate larger roles and impacts of endobacteria in complex natural microbiomes.

In addition to bacteria, fungi can harbor viruses, cyanobacteria, and algae as members of their microbiomes (Kelliher et al. 2023). The group's investigations have identified previously undescribed constituents of the fungal microbiome, including diverse bacterial taxa, archaea, and plant- and algal-derived plastids (Robinson et al. 2021). This discovery of plastids within fungi has led to a JGI Annual CSP award to investigate the mechanistic details underlying their internalization. Researchers have further examined diverse endohyphal microbiome components using multiomics, sequence-based enrichment sequencing techniques, fluorescence *in situ* hybridization imaging, large screens of publicly available sequencing data, and *in vitro* internalization experiments. In conjunction with these efforts, the team has been developing novel bioinformatic pipelines and genomic databases to facilitate screening of fungal sequencing projects for signatures of endohyphal microbiome inhabitants, thus enabling the group and other researchers to expand collective knowledge on the diversity of the fungal microbiome. The group also created a web portal where researchers can run these pipelines, report the relationships that have been found, and compare with existing endohyphal microbiome data (Robinson et al. 2023). Not only is the fungal microbiome much more diverse and complex than previously thought, there is growing interest in examining its role in fungal physiology, fungal evolution, fungal interactions, and fungi in larger communities.

Kelliher, J. M., et al. 2023. "The Endohyphal Microbiome: Current Progress and Challenges for Scaling Down Integrative Multi-Omic Microbiome Research," *Microbiome* **11**, 192.

Longley, R., et al. 2023. "Comparative Genomics of Mollicutes-Related Endobacteria Supports a Late Invasion into Mucoromycota Fungi," *Communications Biology* **6**, 1–13.

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Robinson, A. J., et al. 2021. "Widespread Bacterial Diversity Within the Bacteriome of Fungi," *Communications Biology* **4**, 1168.

Robinson, A. J., et al. 2023. "A Centralized Resource for Bacterial–Fungal Interactions Research," *Fungal Biology* **127**, 1005–09.

Funding Statement: This research was supported by a Science Focus Area grant from the DOE BER program, Biological Systems Science Division, under grant number LANLF59T.

Dissecting Bacterial–Fungal Interactions in the *Bouteloua gracilis* Rhizosphere Microbiome Using Metabolic Phenotyping

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https://genomicscience.energy.gov/lanlbfi/

Project Goals: To understand how root exudates and other carbon compounds shape bacterial–fungal interactions (BFIs) and their roles in ecosystem processes.

Bacteria and fungi play central roles in shaping and facilitating terrestrial ecosystem services and soil functioning. BFIs and interactions with other soil biota are essential to global biogeochemical cycling, soil fertility, and plant health. However, how BFIs influence these functions remains poorly understood. Researchers hypothesize that substrate utilization plays a major role in shaping BFI phenotypes (e.g., antagonistic versus neutral); competition or substrate cross-feeding has the potential to significantly govern the distribution of bacteria and fungi in soils and rhizospheres and influence their roles in biogeochemical cycling, as well as their abilities to associate and colonize plant roots. The work in the BFI Science Focus Area focuses on bacteria and fungi isolated from the heat- and drought-tolerant grass *Bouteloua gracilis* (blue grama) from arid grasslands in the southwestern United States. A framework for the efforts anticipates that blue grama–associated microbes are attracted to roots via root exudates facilitating root colonization, and that blue grama benefits from these interactions (e.g., heat and drought tolerance and pathogen resistance). However, how root exudate composition influences microbial assembly and how microbial competition for root exudates contributes to successful colonization remain poorly understood. Researchers have isolated phylogenetically diverse lineages of the bacterial and fungal members of the blue grama rhizosphere (and root endophytes) and are generating preliminary data on BFI among bacterial–fungal pairs. In combination with genome sequencing, researchers have begun testing representative bacterial and fungal taxa for their potential to utilize a suite of 90 organic compounds using the Biolog Phenotype MicroArrays. Differential substrate utilization profiles document the potential for competition between isolates, indicating that they may have antagonistic interactions *in situ* (i.e., the blue grama rhizosphere). Furthermore, antagonistic interactions may lead to niche partitioning and spatially constrained soil and root colonization. To evaluate the potential for substrate-mediated antagonistic interactions, a key goal for this work is to compare substrate utilization of individual bacterial and fungal isolates, as well as assess the ability of this technology to characterize impacts of BFI in coculture. To do this, bacterial and fungal pairs will be selected based on their individual substrate-utilization profiles, showing similar and contrasting C-source utilization and cocultured in the Biolog plates. While the initial efforts will be focused on carbon (C) utilization, researchers will expand to other nutrients (e.g., phosphorus and nitrogen) in the future. These data provide a foundation for generating new hypotheses for investigating and understanding the ecological roles and impacts of BFI. Using the isolate genomes, next steps will be to generate a predictive understanding of substrate preference and how genetic features can be leveraged to screen for and evaluate BFI in their natural environments, leading to new tools to improve BFI-mediated soil ecosystem services (e.g., plant productivity and C sequestration).

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Assessing Bacterial–Fungal Interactions Across Experimental Scales

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https://sfa-bfi.edgebioinformatics.org/about https://genomicscience.energy.gov/lanlbfi/

Project Goals: To characterize bacterial–fungal interaction (BFI) mechanisms and the impacts of BFIs on their environments under conditions relevant to future climate scenarios.

Bacteria and fungi are often dominant constituents of environmental microbial communities, and interactions between these groups can impact microbial functions within their environments, such as nutrient cycling and plant and soil health. While there have been important advancements in identifying BFIs and their roles, there is still much to be discovered regarding the underlying interaction mechanisms. It is additionally not well understood how these interactions may shift under changing climate conditions and how BFIs may contribute to the resilience of soil communities and plant hosts. Through expansive characterization of BFI across experimental scales, the BFI Science Focus Area team seeks to develop foundational knowledge regarding the drivers of BFI and to provide to the broader research community an integrated suite of publicly available resources as the field rapidly expands. Here, researchers focus on bacteria and fungi isolated from the rhizosphere of the highly stress-tolerant grass, *Bouteloua gracilis,* from the arid grassland sites at the Sevilleta Long Term Ecological Research Station in New Mexico. Grasslands have been estimated to store up to 33% of soil carbon globally (Bai and Cotrufo 2022), and the grassland sites from which the team samples experience stressors relevant to future climate scenarios, such as drought and extreme heat. Researchers hypothesize that microbes associated with *B. gracilis* in the arid grasslands may leverage intermicrobial interactions to respond and adapt to these stressors, providing a useful model ecosystem to understand how BFI will respond as environments become hotter and drier. Using co-occurrence networks built from amplicon sequencing datasets from a large geographical survey, researchers have predicted bacterial and fungal partners that are likely to interact based on co-occurrence rates and which may have greater influence on their ecological contributions due to the prevalence of their interactions. The novel approach will evaluate the ability of co-occurrence models to predict interactions between bacteria and fungi and utilize laboratory-based investigations of interactions to help develop more accurate interaction models based on sequencing and interaction feature data. Several bacterial and fungal isolates were selected for initial laboratory investigations based on network analyses and abundance in sequencing and culture-based surveys. Researchers have conducted preliminary investigations of how environmental conditions, such as nutrient availability and temperature, impact these BFIs. Phenotyping data indicate that some interactions appear to be more strongly impacted by changing environmental conditions, while other interactions are more stable. Researchers have conducted preliminary comparative genomics analyses of genomic differences that may contribute to the distinct responses underlying BFI phenotypes, such as pigmentation and growth. Researchers aim to further characterize the underlying molecular mechanisms using a multiomics approach to identify relevant molecular markers for BFIs, and which may eventually be applied to understanding the relevance of functional features found in broader-scale datasets (e.g., metagenomics and metatranscriptomics). These data will be made publicly available through the Bacterial–Fungal Interactions Portal (https://sfa-bfi.edgebioinformatics.org/about), which was developed to provide a centralized resource of BFI research, including known BFI and their associated studies (Robinson et al 2023).

Bai, Y., and M. F. Cotrufo. 2022. "Grassland Soil Carbon Sequestration: Current Understanding, Challenges, and Solutions," *Science* **377**, 603–608.

Robinson, A., et al. 2023. "A Centralized Resource for Bacterial–Fungal Interactions Research," *Fungal Biology* **127**, 1005–09.

Funding Statement: This research was supported by a Science Focus Area grant from the DOE, BER, Biological Systems Science Division, under grant number LANLF59T.

Science Focus Area
Ecosystems and Networks Integrated with Genes and Molecular Assemblies (ENIGMA)

Towards a Mechanistic Understanding of *Rhodanobacter* Dominance in the Contaminated Subsurface

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http://enigma.lbl.gov

Project Goals: The goal of ENIGMA (Ecosystems and Networks Integrated with Genes and Molecular Assemblies) is to develop theoretical, technological, and scientific approaches to gain a predictive and mechanistic understanding of the biotic and abiotic factors that constrain microbial communities' assembly and activity in dynamic environments. To link genetic, ecological, and environmental factors to the structure and function of microbial communities, ENIGMA uses a systems biology approach to integrate and develop laboratory, field, and computational methods.

Over decades of measurements, *Rhodanobacter* species are consistently dominant in the most contaminated groundwater at the Oak Ridge Field Research Center (ORFRC). Scientists know that *Rhodanobacter* tend to be low pH and high metal-tolerant relative to other bacteria but know less of the precise genetic and physiological mechanisms that enable them to survive and persist in the contaminated subsurface. Here, the team highlights work that exemplifies the ENIGMA Environmental Atlas experimental strategy with the aim of understanding the mechanisms behind *Rhodanobacter* survival at the ORFRC. In particular, high-throughput culturing and biofilm assays reveal phenotypic variability in metal-stress resistance within the *Rhodanobacter* genus and between *Rhodanobacter* and other bacteria from the field site. Researchers have also generated random-barcode transposon sequencing (RB-TNSeq) mutant libraries in multiple ORFRC *Rhodanobacter* strains. Using these high-throughput genetics resources, the team has identified genes important for resistance to the key selective inorganic ion stressors at the ORFRC, including 33 efflux genes important for tolerance to 22 different inorganic ions. In addition, researchers have also implemented the DOE Joint Genome Institute DNA affinity purification sequencing (DAP-seq) approach to examine transcriptionally acting response regulators of signaling systems for two different *Rhodanobacter* strains. Additionally, the project has also developed CRISPR-based tools for precision genetics in ORFRC *Rhodanobacter* strains. Despite these advances, cultivation and analysis of the most highly abundant *Rhodanobacter* strain present in groundwater has remained a challenge. The project has made advances on this front by leveraging long-read metagenomics to identify key traits, such as its unusual genomic capacity for carbon fixation.

The team has also identified an unusually high number of toxin–antitoxin systems in this genome, which may suggest rampant phage infection in the contaminated groundwater. As the project gains more insights into functional genomics and physiology of cultured *Rhodanobacter*, researchers will improve the ability to predict traits in uncultured strains.

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Connecting Microbial Genotype to Phenotype in Bacterial Strains from a Dynamic Subsurface Ecosystem Using ENIGMA 'Environmental Atlas'

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Project Goals: The goal of ENIGMA (Ecosystems and Networks Integrated with Genes and Molecular Assemblies) is to develop theoretical, technological, and scientific approaches to gain a predictive and mechanistic understanding of the biotic and abiotic factors that constrain microbial communities' assembly and activity in dynamic environments. To link genetic, ecological, and environmental factors to the structure and function of microbial communities, ENIGMA uses a systems biology approach to integrate and develop laboratory, field, and computational methods.

The project has made significant progress towards developing an "ENIGMA Environmental Atlas" and mapping genotype to phenotype for a significant number of diverse subsurface microbes from a field site, the Oak Ridge Field Research Center (ORFRC). This Atlas includes a growing collection of close to 3,000 isolates across diverse phyla. Enrichment and isolation efforts reveal that microbial necromass is a major nutrient source for the community, and one recent success includes isolation of novel nitrous oxide reducers from the field site. High-resolution electron microscopy images have revealed unique morphotypes and features of ENIGMA isolates based on growth and nutrient conditions. Researchers are also isolating novel phages and phage tail–like bacteriocins (tailocins) from the field site. The systematic study of bacteria–phage and tailocin interactions will provide novel insights into microbial community dynamics and functional genomics. Genome sequencing of over 1,100 bacterial isolates to date has revealed both macro and microdiversity, such as in *Sediminibacterium* sp., with regards to denitrification genes. To facilitate analyses of these genomes, the team has developed scalable, web-based portals for rapid comparative genomics (https://fast.genomics.lbl.gov/cgi/search.cgi), including those that can readily incorporate newly sequenced genomes (https://iseq.lbl.gov/genomes). Applying diverse high-throughput phenotypic and genome-wide mutant libraries, researchers have investigated the physiology of strains under *in situ* conditions, and results indicate differential phenotypes in outer-membrane genes under transient and chronic metal exposure in *Pantoea* sp. In addition, the team has discovered a novel origin of replication in this strain that allows transformation and expression of non-native genes. Such capability may allow a similar exploration of the metabolism and gene functions in other strains. Here, the team highlights several instances where the ENIGMA Atlas is used to better understand the complexities that govern microbial function in the environment and presents progress on the development of such a unique community-usable platform.

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ENIGMA Environmental Simulations and Modeling: Predictive Modeling and Mechanistic Understanding of Field Observations

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https://enigma.lbl.gov

Project Goals: The goal of ENIGMA (Ecosystems and Networks Integrated with Genes and Molecular Assemblies) is to develop theoretical, technological, and scientific approaches to gain a predictive and mechanistic understanding of the biotic and abiotic factors that constrain microbial communities' assembly and activity in dynamic environments. To link genetic, ecological, and environmental factors to the structure and function of microbial communities, ENIGMA uses a systems biology approach to integrate and develop laboratory, field, and computational methods.

To achieve its project goals, ENIGMA has been organized into several campaigns involving multiple institutes with varying expertise. The overarching goal of the Environmental Simulations and Modeling campaign is to simulate, model, and predict the mechanistic foundations of phenomena observed at a field site, the Oak Ridge Reservation Field Research Center.

Through field surveys and the recently installed SubSurface Observatory, the team collects high temporal-resolution datasets of environmental parameters [e.g., pH, dioxygen (O_2), nitrate, metabolites] and has generated an insightful view of the dynamic nature of the field-site subsurface. By monitoring rainfall events, researchers observed that such events can be followed by a sudden decline in both pH and dissolved O_2, and this transition from a neutral to an acidic pH increases the emissions of nitrous oxide (N_2O). To investigate this phenomenon in the laboratory, the team uses an established synthetic community of two field denitrifiers, *Rhodanobacter* sp. R12 and *Acidovorax* sp. 3H11, which together can perform complete denitrification but cannot independently. Through laboratory simulations utilizing time-course experiments, researchers established that a change in pH from neutral to pH 6 can decouple the denitrification process within the synthetic community, leading to significant increases in N_2O emissions. Additional abiotic controls have shown similar decoupling of denitrification partitioning at varying carbon/nitrogen ratios, oxygen levels, and increased metal concentrations such as nickel. These studies have generated a compendium of 306 transcriptomes that have been used to construct an R12/3H11 gene regulatory network that may help explain and predict how environmental fluctuations at the field site will impact emissions of N_2O.

Given the observed field dynamics, the team has constructed customized drip-flow reactors to mimic ecologically relevant subsurface parameters. The reactor systems generate a vertical O_2 gradient (aerobic to anoxic), mirroring observations from field wells. Additionally, reactors can contain sediment particles to allow for microbial surface attachment or to remain in suspension, reflecting different regimes in subsurface habitats. A five-member synthetic community, comprising facultative anaerobes of varying physiological capabilities that respire nitrate, is used to understand the interplay between attached and planktonic communities across the O_2 gradient. Initial results show distinct stratification of microbial communities along the attached phase of the gradient, suggesting structure–function relationships at the community level. Long-term experiments are underway to probe community stability and the effects of environmental perturbations that simulate field observations.

To further explore the abiotic factors that determine community composition and biogeochemistry at the field site, the project is constructing anaerobic microbiological enrichments under varying nitrate concentrations, carbon sources, and pH conditions. Long-read metagenomic analyses of these enrichments are used to construct community networks relating taxonomy, biogeochemistry, and functional abilities. This information will guide the development of next-generation synthetic communities that recapitulate the natural community assembly process for continued discovery of genetic mechanisms underlying observations from the field.

Funding Statement: This material by ENIGMA, a Science Focus Area at Lawrence Berkeley National Laboratory, is based upon work supported by the DOE Office of Science, BER program, under contract number DE-AC02-05CH11231.

Role of Nitrogen Oxides in a High Nitrate and Heavy Metal–Contaminated Field Site: What Has Been Observed and What Researchers Aim to Understand

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Project Goals: ENIGMA (Ecosystems and Networks Integrated with Genes and Molecular Assemblies) use a systems biology approach to understand the interactions between microbial communities and the ecosystems that they inhabit. To link genetic, ecological, and environmental factors to the structure and function of microbial communities, ENIGMA integrates and develops laboratory, field, and computational methods.

Increasing atmospheric nitrous oxide, a greenhouse gas with 300 times greater radiative trapping than carbon dioxide (CO_2), is primarily attributed to intensive agriculture and the impacts of climate change on soil conditions, estimated to contribute 73% of all U.S. nitrous oxide emissions. Prior studies revealed extremely high fluxes of nitrous oxide from

the saturated subsurface and groundwater within the Field Research Center (FRC) at Oak Ridge National Laboratory, but virtual absence of surface emissions. Ongoing characterization of system microbiota suggested a source derived from the activities of a subsurface community dominated by *Rhodanobacter* species (Carlson et al.) active in the low-pH, high-nitrate groundwater, whereas the microbiota expressing the nitrous oxide reductase in the less contaminated upper soil column functioned as a major sink. These features suggested the utility of this system to better resolve environmental factors controlling nitrous oxide flux, both of its production and consumption. Ongoing studies, using stable isotope analyses and geochemical monitoring, demonstrated active nitrous oxide reduction at pH of 4, well below the observed limit for described industrial and environmental systems. Isotopic compositional studies of nitrogen species suggested that denitrification and chemodenitrification were both major sources of nitrous oxide, with a minor contribution by nitrification in shallower regions supported by the recovery of 16S sequences affiliated with known nitrifiers. A depth-resolved metagenomic analysis of the soil column showed a strong correlation between nitrous oxide depletion in the upper soil and the enrichment of microorganisms encoding the Clade II nitrous oxide reductase, whereas Clade I populations were more abundant near the variably saturated zone in proximity to groundwater. Researchers are now developing instrumentation, isotopic methods, and sampling strategies to confirm the role of the Clade II nitrous oxide variant in suppressing surface nitrous oxide emissions, combining isotopic, geochemical, and molecular measures to identify environmental variables controlling this critical function. Researchers are also quantifying isotopic fractionation and affinity for nitrous oxide of isolates encoding Clade I and II nitrous oxide reductase genes to inform the ecological role of these variants at the site. These studies will be based at the newly installed ENIGMA SubSurface Observatory (Newcomer et al.) at the FRC, providing the opportunity for continuous monitoring of nitrous oxide flux from wells screened at different depths and coupled with geochemical, isotopic, and biological characterization. The goal is to develop a predictive understanding of biological and environmental factors controlling the emission of this critical greenhouse gas.

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Reactive Transport Modeling for Prediction of Nitrous Oxide Emission from the Subsurface Observatory at a Nitrate-Contaminated Site in Response to Rainfall Events

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http://enigma.lbl.gov

Project Goals: Ecosystems and Networks Integrated with Genes and Molecular Assemblies (ENIGMA) uses a systems biology approach to understand the interaction between microbial communities and the ecosystems that they inhabit. To link genetic, ecological, and environmental factors to the structure and function of microbial communities, ENIGMA integrates and develops laboratory, field, and computational methods.

The subsurface environment is one of the major sources of global nitrous oxide (N_2O) emissions. However, the estimation of N_2O from biotic and abiotic pathways in subsurface systems is still poorly understood. Researchers estimated N_2O production by building a field-scale reactive transport model via PFLOTRAN that integrates potential pathways of N-cycling at Area 3, which exhibits high concentrations of nitrate and low pH levels. The Subsurface Observatory (SSO) is located at Area 3 and is an intensive sampling site (5 x 5 x 8 m^3). The SSO was designed and established by the ENIGMA Science Focus Area to provide high temporal-resolution datasets of groundwater flow, chemistry, and microbial communities with a highly instrumented set of continuously monitored, nine-multiport groundwater wells. The heterogeneous permeability field of the SSO site is reconstructed on the basis of the high-resolution data of soil types from Cone Penetration Testing, with fine grid blocks (0.3 x 0.3 x 0.004 m^3) for a variably saturated zone (302.5 to 304.5 m depth) and 0.3 x 0.3 x 0.1 m^3 for the rest of the domain. The flow model has been initially calibrated and

validated from the dataset of rainfall events and groundwater table elevation, collected for a dry season from September to December 2023.

Regarding reactive transport, the reaction network of the model includes many biogeochemical reactions for nitrogen cycling, such as nitrification and denitrification, as well as other reductive processes like iron and sulfate reduction. Based on this wide range of biogeochemical reactions, N_2O emission can be estimated by various biotic and abiotic pathways and calibrated by the measurements (e.g., pH, dissolved oxygen, and nitrate concentration) from the SSO wells. As a result, researchers show the emergence of hot spots and hot moments of N_2O emission at the SSO site under a series of rainfall events.

Lui, L. M., et al. 2021. "Mechanism Across Scales: A Holistic Modeling Framework Integrating Laboratory and Field Studies for Microbial Ecology," *Frontiers in Microbiology* **12**, 642422. DOI:10.3389/fmicb.2021.642422.

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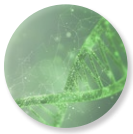

Science Focus Area
Microbial Community Analysis and Functional Evaluation in Soils (m-CAFEs)

Developing Reduced-Complexity Microbial Communities for Editing Across Scales

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https://mcafes.lbl.gov

Project Goals: The program's goal is to understand the interactions, localization, and dynamics of grass rhizosphere microbial communities at the molecular level (e.g., genes, proteins, and metabolites) to enable accurate predictions and interventions to effectively manage and harness microbes to achieve DOE missions in sustainable energy and carbon cycling.

Synthetic communities are excellent tools in microbial ecology research to decipher the complexity in microbe–microbe and plant–microbe interactions. However, this approach often constructs these communities by pooling together individual isolates that are not known to interact or even inhabit the same environment, making the system less biologically relevant. By contrast, reduced complexity communities created using enrichment strategies from native environments can produce less-complex mixtures of naturally occurring and interacting organisms. Using a combination of these natural enrichment communities, genome-resolved metagenomics, and networking microbiome sequencing data, these projects have developed reduced-complexity communities from both field soil and the plant rhizosphere. Communities were enriched on multiple carbon compounds in minimal media conditions to generate different taxa composition from the same soil inoculum, and then subcultured over multiple months to generate a highly stable microbiome. These communities were further tested for freezing tolerance and reproducibility over multiple freeze–growth cycles to confirm community stability under cryogenic conditions and allow for higher predictability of community structure. This high predictability will enable the modeling and precision community editing of native but elusive members of the soil environment, expanding knowledge of biologically relevant interactions in this complex ecosystem. These reduced-complexity communities will be tested in field-simulated conditions to allow for testing microbiome editing capabilities across scales.

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Spatiotemporal Consumer-Resource and Genome-Scale Metabolic Modeling of the Rhizosphere Microbiome

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https://mcafes.lbl.gov/

Project Goals: The goal of the program is to understand the interactions, localization, and dynamics of grass rhizosphere microbial communities at the molecular level (genes, proteins, and metabolites) to enable accurate predictions and interventions to effectively manage and harness microbes to achieve DOE missions in sustainable energy and carbon cycling.

One of the challenges with dealing with the wealth of experimentally generated data in the project is to formulate a theoretical framework with predictive capability. Researchers approach this problem by employing predictive modeling strategies that integrate the increasingly available genomic information with mechanistic and machine learning modeling methodologies.

Our current modeling efforts are focused on two distinct strategies. The first, based on flux balance analysis (FBA), is aimed at pushing the boundaries of completeness and accuracy in simulating the spatiotemporal dynamics of rhizosphere communities. The second, based on experimentally parametrized differential equation models, is aimed at exploring tradeoffs between detail and scalability for increasingly large communities.

For the first strategy, researchers merge genome-scale reconstructions of cellular metabolism, assembled through the DOE Systems Biology Knowledgebase platform (Arkin et al. 2018) and manual curation of specific rhizosphere strains, with a spatiotemporal biophysical model of bacterial propagation implemented in the software COMETS (Computation Of Microbial Ecosystems in Time and Space; Harcombe et al. 2014; Dukovski et al. 2021). COMETS is freely available at runcomets.org. Layouts for specific scenarios can be simulated through a Python macro-language. Researchers are using COMETS to build detailed biophysical models of the rhizosphere microbiome, starting from synthetic communities composed of 17 common rhizosphere microbiome members. These models will help researchers understand the interplay between metabolic and biophysical processes in shaping the spatial organization of microbial biomass metabolism around plant roots, and the nature of microbiome–plant interaction. These COMETS models will be used to simulate effects of genetic modifications, such as gene knockouts, to prioritize experimental community editing targets.

A second strategy is based on the usage of Consumer Resource Modeling (CRM; Mehta and Marsland 2021). This methodology provides a fast way of building coarse-grained models of complex microbial communities, trading detail for scalability and speed and extending ecosystem models to virtually thousands of species (Mehta and Marsland 2021; Silverstein et al. 2023). While CRMs may not be able to accurately capture many nonlinear features of microbial metabolism (such as diauxic shifts) and intracellular perturbations, they provide a valuable theoretical framework for studying complex bacterial communities. Researchers have developed a CRM of a synthetic community composed of the same 17 rhizosphere bacteria being simulated in COMETS. After estimating and optimizing the CRM parameters based on detailed exometabolomics data for individual microbes, researchers show that the CRM can predict pairwise interactions and help map the role of metabolic crossfeeding across species in multispecies consortia. Furthermore, the CRM allowed researchers to predict new communities with maximal diversity.

Future efforts will focus on CRM predictions of environmental perturbations that could drastically alter community structure and dynamics, and comparing COMETS and CRM simulations in an attempt to develop a unified scalable strategy for effective community prediction and editing.

Arkin, A. P., et al. 2018. "KBase: The United States DOE Systems Biology Knowledgebase," *Nature Biotechnology* **36**, 566–69.

Dukovski, I., et al. 2021. "A Metabolic Modeling Platform for the Computation of Microbial Ecosystems in Time and Space (COMETS)," *Nature Protocols* **16**, 5030–82.

Harcombe, W. R., et al. 2014. "Metabolic Resource Allocation in Individual Microbes Determines Ecosystem Interactions and Spatial Dynamics," *Cell Reports* **7**, 1104–15.

Mehta, P., and R. Marsland III. 2021. "Cross-Feeding Shapes both Competition and Cooperation in Microbial Ecosystems," *arXiv*:2110.04965.

Silverstein, M., et al. 2023. "Metabolic Complexity Drives Divergence in Microbial Communities," *BioRxiv.* DOI:10.1101/2023.08.03.551516.

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Leveraging Type I-F CRISPR-Associated Transposase Regulators to Improve Editing Efficiency

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https://mCAFEs.lbl.gov

Project Goals: The goal of this program is to understand the interactions, localization, and dynamics of grass rhizosphere microbial communities at the molecular level (genes, proteins, and metabolites) to enable accurate predictions and interventions to effectively manage and harness microbes to achieve DOE missions in sustainable energy and carbon cycling.

Functional understanding of microbial gene functions is largely based on genetic interrogation of isolated organisms, providing limited insights into the importance of genes within microbial communities, including the rhizosphere, which is the focus of the program. To address these knowledge gaps, recently researchers have created a generalizable toolset for targeted genome editing of individual organisms within complex microbial communities that uses type I-F CRISPR-associated transposons (CASTs) to make targeted genetic edits to complex microbial communities (Rubin et al. 2022). CASTs are broadly dispersed across bacteria and capable of integrating large genomic payloads. However, little is known about the host molecular factors which regulate CAST integration, and their widespread utilization is limited by low editing efficiency across diverse, nonmodel bacteria. To expand the range and applicability of Type I-F CASTs as editing tools, the group employed a genome-wide mutant screening approach to identify putative regulators of CAST transposition in established model systems in which CAST is known to integrate. Candidate regulator hits were individually validated, with a particular focus on well-characterized genes involved in known mechanisms. Next, the team conducted a bioinformatic survey for the conservation of the candidate regulator hits across broad bacterial phyla. Finally, the team leveraged its findings by constructing vectors that incorporate these key regulators to increase editing efficiency. These results will shed light on the molecular mechanisms underlying CAST integration and enable more efficient editing in diverse nonmodel microorganisms. This information will enable the team to extend the application of community editing to better understand the molecular mechanisms governing assembly and interactions in the rhizosphere.

Rubin, B. E., et al. 2022. "Species- and Site-Specific Genome Editing in Complex Bacterial Communities," *Nature Microbiology* **7**, 34–47. DOI:10.1038/s41564-021-01014-7.

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Science Focus Area
Phenotypic Response of the Soil Microbiome to Environmental Perturbations

Metabo-Lipidomics Unveil Root Exudate Molecular Diversity and Functional Impacts on Soil Microbial Communities

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https://www.pnnl.gov/projects/soil-microbiome/research

Project Goals: Pacific Northwest National Laboratory's (PNNL's) Phenotypic Response of Soil Microbiomes Science Focus Area (SFA) aims to achieve a systems-level understanding of the soil microbiome's phenotypic response to changing moisture. Researchers perform multiscale examinations of molecular and ecological interactions occurring within and between members of microbial consortia during organic carbon (C) decomposition, using chitin as a model compound. Integrated experiments address spatial and interkingdom interactions among bacteria, fungi, viruses, and plants that regulate community functions throughout the soil profile. Data are used to parametrize individual- and population-based models for predicting interspecies and interkingdom interactions. Laboratory and field experiments test predictions to reveal individual and community microbial phenotypes. Knowledge gained provides a fundamental understanding of how soil microbes interact to decompose and sequester organic C and enables prediction of how biochemical reaction networks shift in response to changing moisture regimes.

The rhizosphere, where plant roots meet soil, is a hub of biogeochemical activity. The impact of the small molecule metabolites and lipids in root exudates on microbial community structure, gene expression, and processes that control cycling and long-term storage of C are poorly understood. Here, the goal was to discover the molecular chemodiversity of metabolites and lipids in root exudates and root-associated soils to advance the understanding of plant–microbial feedbacks that regulate C cycling. Researchers worked with mature, field-grown tall wheatgrass (*Thinopyrum ponticum*), a deep-rooted perennial plant from the Tall Wheatgrass Irrigation Field Trial in Prosser, Wash., which features marginal, low-carbon aridisols. Researchers optimized exudate collection protocols to enable the capture of nonpolar lipids in addition to polar and semipolar metabolites. Researchers found that rates of C input via hydrophobic exudates were approximately double that of aqueous exudates, and C/N ratios were markedly higher in hydrophobic compared to aqueous exudates (459 ± 90 versus 14.40 ± 0.58), emphasizing the importance of lipids, due to their high carbon content. Researchers used liquid chromatography–coupled tandem mass-spectrometry (LC-MS/MS) for paired untargeted metabolomics and lipidomics or metabo-lipidomics for maximizing molecular coverage. To address the challenge of metabolite annotation, a major bottleneck in metabolomics, the team employed both MS/MS spectral library searching and deep learning–based chemical class assignment. The tandem approach substantially increased the characterization of the chemodiversity of root exudates. Notably, in an unprecedented characterization of intact lipids in root exudates, the team discovered the presence of a diverse variety of lipids, including substantial levels of triacylglycerols (~19 μg/g fresh root per minute), fatty acyls, sphingolipids, sterol lipids, and glycerophospholipids. To understand how the spatial gradient of rhizodeposition impacts microbial community composition and metabolism, researchers performed metabo-lipidomics, metagenomics, and metatranscriptomics on a gradient of soil fractions with varying proximity to the roots. Lipids in exudates and soils had lower nominal oxidation state of C (NOSC) compared to more polar metabolites, suggesting increased persistence and less susceptibility to microbial breakdown. The team observed that microbial expression in members of the Actinomycetota, Acidobacteriota, Bacteroidota, Methylomirabilota, Myxococcota, and Thermoproteota increased close to the root. Nucleoside metabolites (structural components of RNA) were more abundant near the root, suggesting higher microbial activity. Community expression related to the biosynthesis of secondary metabolites and fatty acid degradation increased closest to the roots, while nitrogen metabolism decreased. Focusing on the phyla responsible for these metabolisms, the Actinomycetota and Methylomirabilota had the most significant gradients in abundance as distance toward the root decreased. Triacyglycerols and microbial phospholipids were abundant in bare soil while most secondary metabolites and organic

* Presenting Author

acids increased close to the root. Here, researchers show that metabo-lipidomics enables direct measurements of the functional molecules that govern metabolism, signaling, and resource sharing among microbes and in microbe–plant interactions. This builds on recent work that demonstrated the value of intact lipids in soil ecosystems as sensitive indicators of environmental stress response and substrate availability, and highlights their great potential for interrogating interkingdom interactions and soil C accrual (Couvillion et al. 2023; Naasko et al. 2023).

Couvillion, S. P., et al. 2023. "Rapid Remodeling of the Soil Lipidome in Response to a Drying-Rewetting Event," *Microbiome* **11**(1), 34.

Naasko, K. I., et al. 2023. "Influence of Soil Depth, Irrigation, and Plant Genotype on the Soil Microbiome, Metaphenome, and Carbon Chemistry," *mBio* **14**(5), e01758-01723.

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Model Communities of Soil Microbiomes Reveal Details of Carbon Use Efficiencies and Interkingdom Interactions Across Scales

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https://www.pnnl.gov/projects/soil-microbiome/research

Project Goals: Pacific Northwest National Laboratory's Phenotypic Response of Soil Microbiomes Science Focus Area aims to achieve a systems-level understanding of the soil microbiome's phenotypic response to changing moisture. Researchers perform multiscale examinations of molecular and ecological interactions occurring within and between members of microbial consortia during organic carbon (C) decomposition, using chitin as a model compound. Integrated experiments address spatial and interkingdom interactions among bacteria, fungi, viruses, and plants that regulate community functions throughout the soil profile. Data are used to parametrize individual- and population-based models for predicting interspecies and interkingdom interactions. Lab and field experiments test predictions to reveal individual and community microbial phenotypes. Knowledge gained provides a fundamental understanding of how soil microbes interact to decompose and sequester organic C and enables predictions of how biochemical reaction networks shift in response to changing moisture regimes.

The fate of soil organic C depends in part on how efficiently bacteria and fungi incorporate C into biomass. Higher fungal:bacterial ratios in soil microbiomes have been associated with lower C-use efficiencies (CUE), and CUE is also sensitive to environmental factors, including C source (Soares 2019; Ullah et al. 2021). Therefore, understanding both CUE and interkingdom interactions, and how they affect one another, is key to a complete understanding of the soil ecosystem. However, as these systems are incredibly complex, direct analysis is often difficult. In this project, researchers use a model community of bacterial species, Model Soil Consortium – 2 (MSC-2) to explore how CUE is affected by different experimental growth conditions under four different C sources (N-acetylglucosamine, trehalose, chitin, and carboxymethylcellulose; McClure et al. 2022). Researchers show that faster growth (determined via cell counting) does not always reflect high CUE and that, in certain cases, CUE values are the highest in conditions causing slower or stagnant growth. The team also found that under growth conditions with shaking, vitamin and mineral deficiencies led to slower growth and lower CUE that was dependent on the specific C sources. This suggests that factors that can limit growth (such as lack of key vitamins) are not always the rate-limiting step if another factor (complex C) is present. Identifying a rate-limiting factor can be difficult but the use of model communities helps by discovering paradigms that can be applied to more complex systems.

Researchers extended the successful model community analysis by increasing the complexity to the interkingdom level through the addition of a fungal partner, *Fusarium oxysporum*, and the structural complexity by using glass beads in a spatially structured soil habitat. The team found that the respiration of bacterial and fungal partners is greatly increased when cultured together versus separately, revealing interkingdom interactions that can positively affect community metabolism. Researchers are expanding this work through the development of a Microbial Rhizosphere Community (MRC-1), a community of cocultured bacteria and fungi that will be key to future analysis of how CUE is affected by fungal:bacterial ratios and interactions.

In parallel, researchers are evaluating soils from the Tall Wheatgrass Irrigation Field Trials in Prosser, Wash. The team generated 13 metagenome samples from fungal floats of field soil, from which 333 metagenome-assembled genomes (MAGs) have been derived. These MAGs represent microorganisms, predominantly bacteria, associated with the fungal hyphosphere. Several novel genera were identified, some containing metabolic pathways for the degradation of complex C substances like chitin, cellulose, and starches, which may aid survival in the hyphosphere ecosystem.

The work presented here illuminates several potential crosskingdom interaction events across scales of complexity (soil analogous laboratory systems and field experiments). Future experiments in this area will explore how interactions between species, an approach made simpler with the defined MSC-2 community where species can be removed or added easily, drive CUE. Researchers also propose to use these findings to design and implement experiments that test hypotheses generated in controlled laboratory systems in native field environments so that it can be determined whether and to what degree CUE findings scale across systems.

McClure, R., et al. 2022. "Interaction Networks are Driven by Community-Responsive Phenotypes in a Chitin-Degrading Consortium of Soil Microbes," *Msystems* **7**, e00372-00322.

Soares, M., and J. Rousk. 2019. "Microbial Growth and Carbon Use Efficiency in Soil: Links to Fungal–Bacterial Dominance, SOC-Quality, and Stoichiometry," *Soil Biology and Biochemistry* **131,**195–205.

Ullah, M. R., et al. 2021. "Drought-Induced and Seasonal Variation in Carbon Use Efficiency is Associated with Fungi:Bacteria Ratio and Enzyme Production in a Grassland Ecosystem," *Soil Biology and Biochemistry* **155**,108159.

Funding Statement: Pacific Northwest National Laboratory (PNNL) is a multiprogram national laboratory operated by Battelle for the DOE under contract number DE-AC05-76RLO 1830. This program is supported by the DOE Office of Science, through the Genomic Science Program, BER program, under FWP 70880. A portion of the metagenomic data was provided by the DOE Joint Genome Institute through project BERSS 508623.

Microbial Responses to Scaling Complexity in Chitin Decomposition with Changing Moisture and Structure Levels

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https://www.pnnl.gov/projects/soil-microbiome/research

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The complexity of the soil microbiome and its environment makes it difficult to understand the networks of interactions among community members, ranging from positive interactions, such as metabolite exchange, to negative interactions, like competition. Moisture is a critical attribute of the soil environment that constrains access to resources and interactions within the community, impacting microbial metabolism and biogeochemical processes. Here, the group investigates microbial metabolic interactions and functions that govern organic matter decomposition under contrasting moisture conditions across three levels of biogeochemical complexity that use chitin as a model substrate. At the most reduced complexity, the team used a tractable Model Soil Consortium containing eight members (MSC-2) to understand interspecies interactions governing degradation of chitin in a well-mixed system (McClure et al. 2022). The team expanded to an intermediate-complexity consortium of 31 members (MSC-1) incubated in a spatially structured synthetic soil habitat to identify microbial interactions

and metabolic pathways involved in chitin decomposition (McClure et al. 2020). At the highest level of biogeochemical complexity, researchers used laboratory incubations of soil with chitin amendments collected from the team's Tall Wheatgrass Irrigation Field Trial in Prosser, Wash., to understand how microbial interactions mediate the decomposition of organic substrates. By examining chitin decomposition in a series of experiments that scale in biological and chemical complexity, researchers test how outcomes from culturing experiments translate to soil environments.

Experiments from reduced complexity MSC-2 incubations demonstrate the importance of a subset of chitin degraders for promoting community function. *Streptomyces* was a key member responsible for most chitin degradation, while other organisms like *Dyadobacter* had small, realized niches and low expression. Expanding on this complexity, MSC-1 was used to test impacts of structure and moisture on community interactions in synthetic soil habitats. Using genome-resolved metagenomics and metatranscriptomics, the study shows consistency across experimental scales, with members from MSC-2 retaining prevalent expression patterns in the unstructured broth incubations of MSC-1 (i.e., *Streptomyces)*. In contrast, *Dyadobacter* was a highly active member of broth incubations in MSC-1. Introducing structure invoked significant treatment effects observed as a shift from *Dyadobacter*- and *Streptomyces*-dominated communities in broth to *Ensifer* in structured incubations. These patterns were likely a result of motility- and transporter-related gene expression present in *Ensifer* but not in *Dyadobacter* or *Streptomyces*. Microbial consortium responses to moisture and chitin amendment were tested in a soil-based experiment by screening for chitinolytic and carbon cycling enzymes. Chitin amendment increased chitinolytic response regardless of moisture level. For other carbon cycling enzymes, high moisture caused greater activity compared to low moisture soils. Activity-based probes (ABP) were used to identify organisms producing chitinolytic enzymes. Moisture status and chitin amendment impacted the recovery of chitinolytic genera (*Chitinibacter*, *Cellvibrio*, and *Massilia*) enriched using ABPs, leading to evidence for division of labor on carbon cycling and fitness variation for soil moisture.

These results highlight how genome-resolved multiomics and scaling experimental complexity aid researchers' understanding of microbial communities and suggest a disconnect between broth-based incubations and native incubations of soil. Researchers aim to use this knowledge to move beyond lab-scale experiments and towards integrating *in vivo* experimentation to field-scale *in situ* experiments.

McClure, R., et al. 2020. "Development and Analysis of a Stable, Reduced Complexity Model Soil Microbiome," *Frontiers in Microbiology* **11**, 1987. DOI:10.3389/fmicb.2020.01987.

McClure, R., et al. 2022. "Interaction Networks are Driven by Community-Responsive Phenotypes in a Chitin-Degrading Consortium of Soil Microbes," *mSystems* **7**. DOI:10.1128/msystems.00372-22.

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Science Focus Area
Plant–Microbe Interfaces

Plant–Microbe Interfaces: Microbially Mediated Host Stress Response—Bridging Field and Laboratory Experiments to Gain Insights into the *Populus*–Microbiome Symbiosis Under Abiotic Stress

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https://pmiweb.ornl.gov

Project Goals: The overriding goal of the Oak Ridge National Laboratory (ORNL) Plant–Microbe Interfaces (PMI) Science Focus Area (SFA) is to predictively understand the productive relationship between a plant host and its microbiome based on molecular ane environmentally defined information. *Populus* and its associated microbial community serve as the experimental system for understanding this dynamic, complex multiorganism system. To achieve this goal, the project focuses on: (1) defining the bidirectional progression of molecular and cellular events involved in selecting and maintaining specific, mutualistic *Populus*–microbe interfaces; (2) defining the chemical environment and molecular signals that influence community structure and function; and (3) understanding the dynamic relationship and extrinsic stressors that shape microbiome composition and affect host performance.

Plants are colonized by beneficial microbes that may enhance their resistance to both abiotic and biotic stress, yet the mechanisms underlying these benefits remain largely unexplored. Coupling field observations to laboratory experiments is essential for understanding these microbe-mediated benefits, yet these cross-scale linkages remain a critical knowledge gap. In this study, researchers sampled *Populus trichocarpa* microbiomes across gradients in temperature and precipitation in Oregon and Washington. These sites revealed distinct microbial communities correlated with varying levels of temperature and moisture. To assess the potential benefits conferred by these microbial communities to host plants under thermal stress, whole soil microbiomes from the sites experiencing the coldest and hottest temperatures were transferred to axenic *Populus* tissue culture plants in a greenhouse environment. Notably, poplars receiving the microbiome from the highest-temperature site demonstrated enhanced growth when placed in a high-temperature chamber, suggesting the microbiomes from warmer sites confer greater thermal tolerance to the host plant. Next, the team employed direct plating and flow sorting microbiome isolation approaches to obtain individual isolates from field sites at the thermal extremes. The project will next compare the isolates with taxa enriched in the microbiome of plants demonstrating thermal tolerance to identify potentially beneficial strains. Building on these findings, the research's subsequent phase will utilize individual isolates assembled into *Populus* synthetic communities (SynCom) to dissect the molecular mechanisms underpinning the observed benefits, bridging the gap between field-based microbial ecology and controlled laboratory experiments. This research contributes to an understanding of the complex interactions between plants and their associated microbial communities, and findings have important implications for leveraging these relationships to enhance plant resilience in the face of changing environmental conditions.

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Plant–Microbe Interfaces: Unraveling How Microbial Adaptation Strategies Are Shaped by the Chemical Environment of the *Populus* Rhizosphere

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Project Goals: The overriding goal of the Oak Ridge National Laboratory (ORNL) Plant–Microbe Interfaces (PMI) Science Focus Area is to predictively understand the productive relationship between a plant host and its microbiome based on molecular and environmentally defined information. *Populus* and its associated microbial community serve as the experimental system for understanding this dynamic, complex multiorganism system. To achieve this goal, researchers focus on: (1) defining the bidirectional progression of molecular and cellular events involved in selecting and maintaining specific, mutualistic *Populus*–microbe interfaces; (2) defining the chemical environment and molecular signals that influence community structure and function; and (3) understanding the dynamic relationship and extrinsic stressors that shape microbiome composition and affect host performance.

The rhizosphere microbiome for *Populus* is critically important but factors driving its formation, maintenance, and stability are poorly understood. This interkingdom interaction is connected and controlled by molecular information, primarily metabolites, and proteins exchanged between the two systems. As such, these biomolecules are key indicators of the dynamic and spatial functioning of these interactions. Based on this need, researchers have optimized liquid chromatography–tandem mass spectrometry approaches for deep, agnostic measurement of microbial proteomes and metabolomes in the rhizosphere in an effort to explore how bacteria adjust their metabolic activities in response to the chemical environment of the *Populus* rhizosphere.

In order to explore the fundamental interactions of the plant microbiome, it is advantageous to employ a lab-based system that has lower complexity than field-based systems. To this end, researchers have examined the interactions of a 10-member customized bacterial community with plant roots, all maintained in an agarose gel plate (Appidi et al. 2022). This enables the detection of microbial proteomes and metabolomes in a temporal and spatial fashion as the microbes encounter and interact with the plant. By employing a metaproteomic approach, researchers contrasted how each of these microbes responds to the plant when grown individually versus how the microbial consortium responds to the plant as a community. Researchers found that while the microbial response is highly individualized, there are some common responses between the isolates and the microbial community that were shared, including the upregulation of proteins associated with chemotaxis, motility, transporters, and metabolism. Metabolomic measurements revealed a large array of primary and secondary metabolites, many of which vary temporally in the plant–microbiome system. Workflows, such as Compound Discoverer, are being used for putative molecular identifications, although many detected compounds elude annotation. One approach to deal with the molecular complexity is to employ molecular networking approaches, such as Global Natural Product Social Molecular Networking (GNPS), to connect similar fragmentation spectra. This enables experimental tandem mass spectrometry (MS/MS) spectra to be matched against a large collection of publicly accessible natural product and metabolomics fragmentation data in the GNPS-community spectral library to assign putative annotations and identify molecular families, which are defined as related MS/MS spectra differing by simple structural or chemical transformations.

The key information obtained above will provide the springboard to extend to the more complex field systems. To address the expected challenges, researchers have focused attention on key advances in sample preparation techniques (to reduce complexity in the samples), MS measurement features, and bioinformatic data mining (Carper et al. 2022). The most recent work has been to evaluate the newly emerging series of commercially available rapid-scanning mass spectrometers. In particular, researchers evaluated a synthetic microbial community sample on the new ThermoFisher Astral MS platform, which scans at least 20 times faster than existing instrumentation. As expected, even with the same sample preparation process, the measurement metrics are about an order of magnitude improved, which equates to greater microbial proteome coverage. This experimental platform should dramatically advance the utility of examining more complex *in situ* microbiomes from field-sampling campaigns and thus provide more detailed information about plant–microbial interactions at the environmental level.

Appidi, A. R., et al. 2022. "Development of an Experimental Approach to Achieve Spatially Resolved Plant Root-Associated Metaproteomics Using an Agar-Plate System," *Molecular Plant Microbe Interactions* **35**(8) 639–49. DOI:10.1094/MPMI-01-22-0011-TA.

Carper, D. L., et al. 2022. "The Promises, Challenges, and Opportunities of Omics for Studying the Plant Holobiont," *Microorganisms* **10**(10), 2013. DOI:10.3390/microorganisms10102013.

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Plant–Microbe Interfaces: Defining Quorum Sensing Signal Potential in the *Populus* Microbiome and Examining its Role in Community Selection and Structure

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Microbial communities play an integral role in the health and survival of their plant hosts. Environmental and host factors drive microbial community structure in the rhizosphere, but microbe–microbe chemical signaling undoubtably has a role in structuring the microbial community. The impact of microbe–microbe interactions on multispecies community structure and dynamics is not well understood. Researchers previously established that acyl-homoserine lactone (AHL), quorum sensing (QS), and natural product biosynthesis genes are prevalent in the *Populus* microbiome. QS often regulates extracellular enzyme production, biofilm formation, competence, and secondary metabolite production contributing to microbe–microbe interactions. Here, the group utilized a synthetic biology approach to explore the diversity of AHL signals and an approach based on synthetic communities (SynCom) to investigate the influence of QS on microbial community structure and dynamics.

Bioinformatic analyses identified a large amount of unexplored AHL signal synthase gene (LuxI) diversity in the *Populus* metagenome and bacterial isolate genomes. As part of a DOE Joint Genome Institute (JGI) DNA Synthesis Community Sequencing Project, researchers selected 140 representative undefined LuxI homologs from the *Populus* microbiome for DNA synthesis and expression in the heterologous host *Escherichia. coli*. The group identified AHL signals synthesized by these LuxI homologs in *E. coli* using the JGI nonpolar metabolomics pipeline and mass query language analyses. Researchers detected AHL production in about half of the synthesized LuxI homologs, including the first well-described AHL signal structures for several genera (*Bosea, Duganella, Janthinobacterium, Massilia, Novosphingobium, Rhodanobacter, Rhodoferax, Sphingobium, Sphingomonas, Sphingopyxis,* and *Variovorax*). Interestingly, the predicted AHL signal inventory of the endosphere is distinct compared to that of the rhizosphere/soil and is dominated by LuxI homologs that synthesize atypical AHL signals.

Next, the team utilized a AiiA-lactonase QS-off method with a previously established SynCom to assess the effects of AHL inactivation on microbial community structure, pairwise interactions, biofilm formation, and secondary metabolite production. Preliminary results demonstrate disruption of AHL signaling leads to changes in the community structure. Current efforts are focused on elucidating the molecular mechanisms through which AHL signaling mediates microbial community assembly. Collectively, these diverse applications of metagenomics and cultured representatives of *Populus'* microbial community are facilitating researchers' understanding of how *Populus* selects microbial partners and how its microbiome is structured.

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Plant–Microbe Interfaces: Specialized Fungal Metabolites Regulate Synthetic Fungal Communities and Their Interactions with *Populus*

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Ectomycorrhizal fungi (EMF) are beneficial fungi that colonize the root tissues of multiple host plants. The interactions between EMF and their host are likely governed by metabolites, which act as direct lines of communication between organisms in the rhizosphere. However, the metabolites or signals that are produced when a fungus is alone or with a host or with other microbes are understudied. This team's goal is to identify and characterize the repertoire of metabolites produced when EMFs are in coculture with each other or when colonizing different *Populus* genotypes in tissue culture assays. Firstly, to develop a functioning EMF synthetic community, the group conducted coculture assays between five EMFs isolated from *Populus* roots or within a *Populus* plantation. The aim was to find a combination of EMF that produce a repertoire of metabolites that promote growth of other fungal members. Since it is known that nutrient availability influences metabolite production (Rush et al. 2022; Meena et al. 2023), researchers utilized four different substrate media, two nutrient-rich and two nutrient-deprived. The group's preliminary results determined that *Hebeloma brunneifolium* promoted the growth of nearly all the co-occurring fungi in a nutrient-poor environment; however, this was not reciprocal, with no growth benefit of *H. brunneifolium*. *Laccaria bicolor* and *Cenococcum geophilum,* regardless of the media, had the most beneficial interactions with co-occurring fungi. Based on the above results, the team determined that *C. geophilum, H. brunneifolium,* and *L. bicolor* would benefit each other for fungal growth.

Next, researchers examined how these EMFs individually impact *Populus* root and leaf development over time. Researchers used tissue cultures of *Populus tremula* x *alba* genotype 717-1B4 and *P. trichocarpa* x *P. deltoides* hybrid 52-225. Group members measured plant physiological traits (root growth and leaf development) as well as omic data (volatile organic compounds, metabolomics, and proteomics) to determine the effects of each EMF on *Populus*. After 5 weeks of colonization, the team's preliminary results determined that *C. geophilum* effectively colonized the root, as shown by the development of a Hartig Net, and increased root length and branching. *H. brunneifolium* interestingly did not colonize the host plant but still had a positive growth effect on root and shoot tissue, possibly attributed to volatile organic compounds or specialized metabolites. Lastly, *L. bicolor* effectively colonizes *Populus* tissue culture plants but showed no beneficial tradeoff with its host. Altogether, this project's results show that researchers can construct a synthetic EMF community that will be symbiotic with each other and likely have a positive phenotypic effect on the host plant.

Meena, M. G., et al. 2023. "A Glimpse into the Fungal Metabolomic Abyss: Novel Network Analysis Reveals Relationship Between Exogenous Compounds and Their Outputs," *PNAS Nexus* **2**(10). DOI:10.1093/pnasnexus/pgad322.

Rush, T. A., et al. 2022. "Lipo-Chitooligosaccharides Induce Specialized Fungal Metabolite Profiles that Modulate Bacterial Growth," *mSystems* **7**(6). DOI:10.1128/msystems.01052-22.

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Plant–Microbe Interfaces: Disentangling Microbial-Mediated Plant Stress Tolerance with Synthetic Communities and Automated Phenotyping

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Recent studies have shown that microbes from extreme environments can confer plant stress tolerance. Such studies have led to the hypothesis that microbiomes adapted to harsh environmental conditions can benefit host plants in similar environments. However, the magnitude of these benefits, underlying microbial dynamics, and driving genetic mechanisms remain unclear. The current study employs synthetic community (SynCom) approaches, paired with high-throughput phenotyping and physiological assays, to dissect the specific roles of microbial strains and communities in plant thermotolerance. To quantify microbial benefits on plant growth and physiology across temperatures, SynComs were constructed with selected bacteria and applied to axenic tissue culture *Populus trichocarpa* x *deltoides* within a calcined clay medium. Bacteria were selected and prioritized based on phylotyping data from PMI field sites. The SynCom–host systems were then exposed to a range of temperatures (9°C to 28°C). These systems were enclosed for 3 weeks to ensure community establishment, and then containers were opened for an additional 4 weeks and subjected to automated phenotyping. The addition of a single *Variovorax* bacterial strain significantly enhanced plant growth and photosynthetic efficiency. Further investigation using a heterologous quantitative trait loci study identified a seven-gene interval associated with microbially conferred thermotolerance. Interestingly, genetic analysis revealed a proteosome interacting protein essential for the plant to benefit from the *Variovorax* strain. Future studies are integrating this newly found microbially mediated abiotic stress response pathway with known induced system resistance (ISR) and systemic acquired resistance (SAR) pathways. This knowledge paves the way for developing climate-resilient plants by harnessing the power of beneficial microbes and genetics.

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Science Focus Area
Microbes Persist: Systems Biology of the Soil Microbiome

Unraveling Microbial and Viral Responses to Wetting Using Multiomics

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https://sc-programs.llnl.gov/biological-and-environmental-research-at-llnl/soil-microbiome

Project Goals: Microorganisms play key roles in soil carbon (C) turnover and stabilization of persistent organic matter via their metabolic activities, cellular biochemistry, and extracellular products. Microbial residues are the primary ingredients in soil organic matter, a pool critical to Earth's soil health and climate. The team hypothesizes that microbial cellular chemistry, functional potential, and ecophysiology fundamentally shape soil C persistence, and researchers are characterizing this via stable isotope probing of genome-resolved metagenomes and viromes. The project focuses on soil moisture as a "master controller" of microbial activity and mortality, since altered precipitation regimes are predicted across the temperate United States. The Science Focus Area's (SFA) ultimate goal is to determine how microbial soil ecophysiology, population dynamics, and microbe–mineral–organic matter interactions regulate the persistence of microbial residues under changing moisture regimes.

The intensity and timing of precipitation not only affects soil microbial community composition and microbial ecological strategies, but also microbial-controlled decomposition and soil carbon dioxide efflux. However, scientists do not have a mechanistic understanding of how altered soil moisture regimes will affect soil C persistence or microbial population dynamics. Conducting a wet-up experiment on soils previously subjected to either historical average precipitation (100%) or a 50% water reduction, researchers employed a multiomics approach (16S-quantitative stable-isotope probing, metagenomics, viromics, metatranscriptomics, metabolomics, and lipidomics) to elucidate the mechanisms governing microbial response following wet-up.

Determining the relationship between bacterial traits and life strategies is a crucial step in linking soil microbial communities to ecosystem function. Genomic traits, such as genome size, codon usage, and nucleotide selection, may be particularly useful trait dimensions as they are relatively easy to measure and provide insight into the evolutionary forces which shape bacteria. In response to rewetting, researchers found that metagenome-assembled genomes (MAGs) with high levels of codon bias and cheaper nucleotides in their ribosomal protein genes had high growth rates shortly after rewetting. The team also found that MAGs with smaller genomes had higher growth rates than larger genomes one week post rewetting. Together, these results point towards a set of genomic characteristics that are potentially important for bacteria in highly dynamic soil environments.

The project found that legacy precipitation (50 versus 100% mean annual precipitation; MAP) had the largest impact on microbial population dynamics, where 100% MAP was associated with higher overall growth and mortality rates and greater changes in gene expression across multiple metabolic pathways. Similarly, metabolomics data showed a greater magnitude of changes in metabolite composition after rewetting at 100% MAP. Results suggest that decreased legacy moisture leads to decreased metabolic and growth potential for microbes following drydown, impacting which taxa are able to rapidly respond to rewetting.

The team posits that interactions between the soil water cycle, nutrient availability, and microbial predators (bacterivores and viruses) may also control a significant proportion of soil C flux, but this nascent research area is another major knowledge gap. Researchers detected 172 protist taxa and found that average population growth at 80% field capacity was 4.4 times greater than at 20% field capacity (3.2% versus 0.15% per day, respectively), suggesting that protists' growth activity is highly sensitive to soil moisture. Further, researchers found soil viruses exhibited sensitivity to nutrient availability upon rewetting. The introduction of

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phosphorus diminished the overall richness of RNA viruses, yet it concurrently triggered an upsurge in specific bacterial viruses. The increase of bacterial viruses may be attributed to the alleviation of stress experienced by bacterial hosts due to phosphorus limitation. Conversely, fungal viruses exhibited no significant change, potentially indicating the resilience of fungal mycelial networks in nutrient movement.

In summary, the project identified important genome-level traits predictive of microbial growth response to rewetting. The team also found that differences in legacy precipitation can influence microbial activities long after changes in soil moisture are no longer detectable, and microbial predators are sensitive to changes in soil moisture and nutrient changes.

Funding Statement: This research is based upon work of the Lawrence Livermore National Laboratory (LLNL) "Microbes Persist" Soil Microbiome SFA, supported by the DOE Office of Science, BER program's GSP under award number SCW1632 to LLNL and subcontracts to Northern Arizona University, University of California–Berkeley, and the Pacific Northwest National Laboratory. Work at LLNL was performed under DOE contract DE-AC52-07NA27344.

How Soils Work

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https://sc-programs.llnl.gov/biological-and-environmental-research-at-llnl/soil-microbiome

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Ecophysiology and community ecology are natural stops on the road from genes to ecosystems, are central to understanding how microbes function, respond to substrate availability and environmental changes, release carbon dioxide, consume and produce soil organic matter, and are essential to develop a molecular and mechanistic understanding of microbes' roles in soil C cycling.

Metatranscriptomics is a powerful and relatively new tool to study soil communities. However, the vast number of genes and transcripts and the complexity of their functions and regulation often limit a straightforward interpretation, making it difficult to draw clear conclusions from experiments conducted in natural soils. Instead of a whole transcriptome analysis, the project advocates a modular approach to study ecophysiology and community ecology of wild soil microbial communities. Each module consists of sets of genes related to specific ecophysiological (e.g., starvation responses) or community ecological (e.g., trophic dynamics) functions, founded on expert knowledge applied, criticized, improved, and expanded by researchers using cross-ecosystem comparisons.

As an illustration, the team has analyzed four metatranscriptome datasets to answer the following research questions.

1. It is often assumed that soil microbes spend most of their existence in a state of starvation with high-maintenance energy demand, where substrate is used only for energy production (Hagerty et al. 2018). Is there evidence that transcription of energy production and biosynthesis varies?
2. Although microbes are mostly C-limited, low availability of inorganic nutrients may restrict microbial activity when excess organic C is available. In response to nutrient limitations, microbial cells increase transcription of transporters for the most limiting nutrient (Ishige et al. 2003; Silberbach et al. 2005). Do researchers see signs of inorganic nutrient limitations in metatranscriptome datasets?
3. Bacteria form biofilms consisting of extracellular polysaccharides mixed with lipids, proteins, and DNA. These biofilms protect the encapsulated cells, facilitate their survival, and may have an important role in soil organic matter formation. Can researchers use metatranscriptomes to study when and where biofilms are being produced?

4. Using metatranscriptomes, can researchers detect the influence of carbohydrates on microbial metabolism by looking at gene expression associated with glycolysis versus gluconeogenesis?
5. In addition to being waystations towards a mechanistic perspective of ecosystems, ecophysiology can be a research target by itself.
6. Can researchers use metatranscriptomes to study the molecular mechanisms of regulation of growth and starvation in soil microbial communities?

The experiments compared consist of a glucose addition; a water addition after a seasonal drought; a warming by time-since-deglaciation experiment in Antarctica; and a comparison between bulk, detritosphere, and rhizosphere communities.

Results show that transcript abundances for biosynthesis are mostly proportional to transcript abundances for energy production; high transcript abundances for nitrogen and phosphate transporters indicate short periods of nutrient limitations; and temporally and spatially explicit patterns of growth and biofilm production suggest that progress can be made to resolve questions relating soil organic matter formation and microbial activities.

Overall, the project signals a growing need for biochemical and cell physiological expertise within soil ecology.

Hagerty, S. B., et al. 2018. "Evaluating Soil Microbial Carbon Use Efficiency Explicitly as a Function of Cellular Processes: Implications for Measurements and Models," *Biogeochemistry* **140**, 269–83.

Ishige, T., et al. 2003. "The Phosphate Starvation Stimulon of *Corynebacterium glutamicum* Determined by DNA Microarray Analyses," *Journal of Bacteriology* **185**, 4519–29.

Silberbach, M., et al. 2005. "DNA Microarray Analysis of the Nitrogen Starvation Response of *Corynebacterium glutamicum*," *Journal of Bacteriology* **119**, 357–67.

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Drought Influences Microbial Activity and the Accrual and Composition of Soil Organic Carbon

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Soil microorganisms shape the global carbon balance by transforming plant rhizodeposits and root detritus into soil organic matter (SOM). It remains unclear how drought influences transformations of these distinct root sources, particularly into the largest and slowest-cycling SOM pool mineral-associated organic matter (MAOM), and how researchers might predict these transformations with changing climate. Since living and decaying roots often exist in close proximity, researchers need to understand how their interactions affect the accrual of MAOM (e.g.,

through priming effects induced by enhanced microbial activity or the effects of specific microbial taxa). To investigate relationships between drought, microbial ecophysiology, and SOM accrual in a Mediterranean grassland soil, the team conducted a 12-week continuous $^{13}CO_2$ tracer study with the annual grass *Avena barbata*, tracking movement of rhizodeposits and root detritus into microbial communities and SOM pools under moisture replete (15 ± 4.2%) or water-limited (8 ± 2%) conditions. Upon harvest, the team measured formation of ^{13}C-MAOM from either ^{13}C-enriched rhizodeposition alone, decomposing ^{13}C-enriched root detritus alone, or the two together. The team measured active microbial community composition (via ^{18}O- and 13C-quantiative stable isotope probing; qSIP), microbial community-level growth rate and carbon use efficiency, and the chemical composition of SOM using mass spectrometry. These data inform the modeling, which integrates dynamic plant growth models, microhabitats, and a trait-based dynamic energy budget model (DEBmicroTrait) to simulate how precipitation patterns impact both the timing and magnitude of belowground carbon allocation in A. *barbata*, rhizosphere community dynamics, and ultimately MAOM accrual.

Overall, drought significantly reduced the accrual of ^{13}C-MAOM, with contrasting interactions between habitat and time. In droughted rhizosphere soil, there was significantly less ^{13}C-MAOM relative to moisture-replete soil at week 12. But at that time, moisture-replete rhizosphere soils had the greatest aboveground plant biomass, microbial community-level growth rate, and carbon use efficiency. In the detritusphere, droughted soils had the greatest difference in ^{13}C-MAOM at week 4, during early stages of root litter decomposition. At this same point, detritusphere microbial community–level growth rate and carbon use efficiency was greatest under normal moisture conditions. The chemical composition of SOM was also significantly different between rhizosphere and detritusphere habitats, with greater abundance of diacylglycerophosphocholine lipids in the detritusphere and triacylglycerol lipids in the rhizosphere. Metabolomics suggested more short-chain saturated and hydroxy fatty acids in the rhizosphere and more di- and trisaccharides, C-8 amino sugars, and some nucleobases (thymine and cytosine) in the detritusphere.

When living and dead roots co-existed, the presence of living roots decreased the accrual of ^{13}C-MAOM formed from detritus. However, the inverse was not true: root detritus did not affect ^{13}C-MAOM derived from rhizodeposition. In the detritusphere, the effects of living roots on microbial growth rates depended on soil moisture. Under drought, living roots increased relative growth rates of fungal and bacterial taxa. When soils were moisture replete, living roots increased relative growth rates of detritusphere fungal taxa, with no effect on bacteria. In comparison, the presence of detritus increased relative growth rates of fungal and bacterial rhizosphere taxa regardless of soil moisture.

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Microbes Persist: Towards Quantitative Theory-Based Predictions of Soil Microbial Fitness, Interaction, and Function in Knowledgebase

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https://sc-programs.llnl.gov/soil-microbiome

Project Goals: Microorganisms play key roles in soil carbon (C) turnover and stabilization of persistent organic matter via their metabolic activities, cellular biochemistry, and extracellular products. Microbial residues are the primary ingredients in soil organic matter, a pool critical to Earth's soil health and climate. The team hypothesizes that microbial cellular chemistry, functional potential, and ecophysiology fundamentally shape soil C persistence, and the researchers are characterizing this via stable isotope probing (SIP) of genome-resolved metagenomes and viromes. This study focuses on soil moisture as a "master controller'" of microbial activity and mortality since altered precipitation regimes are predicted across the temperate United States. The Science Focus Area's (SFA's) ultimate goal is to determine how microbial soil ecophysiology, population dynamics, and microbe–mineral–organic matter interactions regulate the persistence of microbial residues under changing moisture regimes.

Researchers have developed a genomes-to-traits workflow (microTrait) and a compatible dynamic energy budget

trait-based model (DEBmicroTrait) to: (1) infer ecologically relevant traits from microbial genomes; (2) systematically reduce the high dimensionality of genome-level microbial trait data by inferring functional guilds (sets of organisms performing the same ecological function irrespective of their phylogenetic origin); (3) quantify within-guild trait variance and capture trait linkages in trait-based models; and (4) explore trait-based simulations under different scenarios with varying levels of microbial community and environmental complexity (Karaoz and Brodie 2022; Marschmann et al. 2024). This computational workflow allows the team to predict tradeoffs involving metabolic, biophysical, and thermodynamic traits of microorganisms. This includes the capability to predict substrate uptake kinetics for broad substrate classes. Researchers are working to integrate this tool within the DOE Systems Biology Knowledgebase (KBase), which will support ongoing efforts to integrate genome-centric knowledge into biogeochemical models.

In addition, the SFA team has formalized and optimized the code for quantitative stable isotope probing (qSIP) into an R package (qSIP2) and documentation website (Hungate et al. 2015; Koch et al. 2018; Simpson et al. 2023). The qSIP2 workflow (and forthcoming KBase applications) allow for the identification of enriched taxa in isotope addition experiments given density fractionation of DNA, sequence counts, and a quantitative measure of abundance in each fraction (e.g., 16S rRNA). The qSIP2 workflow can accept input for both amplicon (e.g., 16S rRNA) and metagenomic (e.g., MAG coverage) data and produce an excess atom fraction enrichment value quantifying the extent of "heavy" isotope incorporation. Results from qSIP can help experimentally identify microbial traits via quantifying the use of a given substrate.

Ongoing work to combine both the qSIP and DEBmicroTrait tools within KBase will provide a strong foundation for researchers who wish to use quantitative *in situ* measurements of microbial ecophysiology and population dynamics to benchmark models and build a predictive understanding of biological processes controlling material fluxes in complex environments.

Hungate, B.A., et al., 2015. "Quantitative Microbial Ecology Through Stable Isotope Probing," *Applied and Environmental Microbiology* **81**, 7570–81.

Karaoz, U., and E. L. Brodie. 2022. "MicroTrait: a Toolset for a Trait-Based Representation of Microbial Genomes," *Frontiers in Bioinformatics* 918853.

Koch, B. J., et al. 2018. "Estimating Taxon-Specific Population Dynamics in Intact Microbial Communities," *Ecosphere* **9**, e02090.

Marschmann, G.L., et al. 2024. "Predictions of Rhizosphere Microbiome Dynamics with a Genome-Informed and Trait-Based Energy Budget Model," *Nature Microbiology* **9**, 421–433.

Simpson, A., et al. 2023. "A Data Standard for the Reuse and Reproducibility of Any Stable Isotope Probing-Derived Nucleic Acid Sequence (MISIP)," *bioRxiv* **2023**(7).

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Early Career

Mapping Perturbations in a Naturally Evolved Fungal Garden Microbial Consortium

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Project Goals: This early career research project is dedicated to achieving transformative molecular-level insights into microbial lignocellulose deconstruction through the comprehensive and informative view of underlying biological pathways provided by the integration of spatiotemporal multiomic measurements (i.e., proteomics, metabolomics, and lipidomics). A focus of this project is to uncover the mechanisms that drive cooperative fungal–bacterial interactions resulting in degradation of lignocellulosic plant material in the leafcutter ant fungal garden ecosystem. This approach will provide the knowledge needed for a predictive systems-level understanding of fungal–bacterial metabolic and signaling interactions that occur during cellulose deconstruction in an efficient, natural ecosystem.

Understanding interkingdom interactions is critical for predicting the metabolic outcomes of environmental perturbations to microbial processes. Biological samples, however, are often complex and heterogeneous. Thus, it is challenging to detect spatial and temporal variation in microbial interactions and activities.

In this study, the research team used six independent naturally evolved leafcutter ant fungal garden consortia that are biologically complex and known to achieve active lignocellulose degradation primarily mediated by *Leucoagaricus* (Khadempour et al. 2021) as the model system. A pathogenic fungus, *Escovopsis*, was introduced to one side of each consortium and proliferated toward its middle section. This introduction created an infection gradient as well as contrasting microbial compositions on the two sides of the consortium. To elucidate the active interkingdom interactions and their metabolic outcomes within this dynamic system, the team applied multiomics (i.e., metaproteomics, lipidomics, and metabolomics) integrated with microscale imaging to capture shifts in microbial community members and map their detected activities (Veličković et al. 2024).

Deep metaproteomics reveal microbial population and functional dynamics along the infection gradient. High selectivity and sensitivity in peptide identifications was achieved with a Thermo Fisher Orbitrap Eclipse Tribrid mass spectrometer equipped with a front-end High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) interface. The research leveraged a reference database containing 50 million proteins of known consortium members, which were grouped into >24 million clusters based on sequence similarity, to annotate the high-resolution tandem mass spectrometry spectra with stringent matching criteria. A total of 263,404 clusters were detected in the metaproteome data with relatively high representations of fungal, bacterial, and plant proteins followed by archaeal and insect proteins.

To identify patterns in the omics data, the team developed a method that leverages unsupervised machine learning algorithms to automatically recognize regularities across the infection gradient. Over 400 metabolites and 500 lipids exhibited significant trends (adjusted p-value<0.05). Combining these trends with the metaproteome data provided pathway-level species-specific interaction patterns. These methods recognized antagonistic interaction patterns between native and pathogenic fungal species. *Leucoagaricus* metabolic activities associated with carbohydrate metabolism and secondary metabolite biosynthesis decreased with increasing *Escovopsis* infection. Bacterial metabolic activities increased with fungal infections in some of the consortia, suggesting an active community response and potential interkingdom interactions under the impact of fungal infection. Interactions unique to the infection interface mapped complex activities underpinning both attack and defense metabolic strategies utilized by consortium members.

These spatiotemporal multiomics measurements provided an integrated road map to efficiently harness microbiome data for a better understanding of microbial interactions and community response to a perturbation. In addition, the measurements provided a predictive systems-level understanding of how symbiotic fungal–bacterial metabolic and signaling interactions enable the fungal garden ecosystem to thrive and degrade lignocellulose in dynamic environments.

Khadempour, L., et al. 2021. "From Plants to Ants: Fungal Modification of Leaf Lipids for Nutrition and Communication in the Leaf-Cutter Ant Fungal Garden Ecosystem," *mSystems* **6**(2). DOI:10.1128/mSystems.01307-20.

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Veličković, M., et al. 2024. "Mapping Microhabitats of Lignocellulose Decomposition by a Microbial Consortium," *Nature Chemical Biology*. DOI:10.1038/s41589-023-01536-7.

Funding Statement: The Pacific Northwest National Laboratory (PNNL) is a multiprogram national laboratory operated by Battelle for DOE under contract DE-AC05-76RL01830. This program is supported by DOE, BER, under the Early Career Award Program. A portion of this work was performed in the William R. Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by BER and located at PNNL.

Interkingdom Interactions in the Mycorrhizal Hyphosphere and Ramifications for Soil Carbon Cycling

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Project Goals: Arbuscular mycorrhizal fungi (AMF) are ancient symbionts that form root associations with most plants. AMF play an important role in global nutrient and carbon (C) cycles, and understanding their biology is crucial to predict how C is stored and released from soil. This Early Career research investigates the mechanisms that underpin synergistic interactions between AMF and microbes that drive nitrogen (N) and C cycling, addressing DOE's mission to understand and predict the roles of microbes in Earth's nutrient cycles. By coupling isotope-enabled technologies with next-generation DNA sequencing techniques, the project investigates soil microbial interactions *in situ* using natural levels of soil complexity. This work will provide a greater mechanistic understanding needed to determine how mycorrhizal fungi influence organic matter decomposition and will shed light on nutrient cycling processes in terrestrial ecosystems.

The arbuscular mycorrhizal association between Glomeromycota fungi and land plants is ancient and widespread; 72% of all land plants form symbiotic associations with AMF. While AMF are obligate symbionts that depend on host plants for C and cannot decompose soil organic matter (SOM), AMF can stimulate the decomposition of SOM and dead plant tissue. Prior Early Career Program research strongly suggests that AMF partner with their microbiome in the zone surrounding hyphae (or "hyphosphere") to encourage decomposition (Nuccio et al. 2022). The team examined AMF–microbial interactions in reduced-complexity microcosms and the field to assess the impacts of AMF on terrestrial C and N cycling processes. In the laboratory, researchers are assessing how AMF and their microbiomes impact litter decomposition, while in the field researchers assess how a deeply rooted perennial grass alters the "zone of influence" of AMF in soil depth profiles.

The molecular mechanisms that underpin interactions between AMF and the microbial community during SOM decomposition is a key knowledge gap. To facilitate metabolomics and mechanistic studies of the hyphosphere, the team has developed the MycoChip, a sterile plant-mycorrhizal microcosm for interrogating hyphal–microbial interactions *in situ*. The MycoChip allows both destructive and nondestructive resampling of hyphosphere communities over time and is optically clear to permit microscopy. This system has two chambers separated by a "raised air-gap" containing a dam to prevent solute exchange between chambers and flanked by mesh barriers to block root entry and create a hyphosphere chamber isolated from the rhizosphere. Researchers used the MycoChip to examine how a living microbiome alters AMF exudation and the exometabolome during SOM decomposition. AMF were either allowed or denied access to nutrients (plant litter and bone meal) in the hyphal chamber by using different mesh sizes (31 μm and 0.45 μm, respectively). To investigate how fungal–microbial interactions impact decomposition, AMF were exposed to "live" versus "dead" soil in the hyphal chamber. Data analysis is ongoing to investigate how AMF impacts the detritusphere metabolome, microbiome, and plant photosynthesis and growth.

Most knowledge about physiology and ecology of AMF (and most soil organisms) has been learned from surface soils that are less than 20 cm deep. In a national field study, researchers assessed how the rhizosphere of a deep-rooted perennial bioenergy grass—switchgrass (*Panicum virgatum*)—alters the "zone of influence" of AMF in depth profiles and impacts soil C stocks. Rhizosphere and bulk samples from paired switchgrass and shallow-rooted fields were collected from 2.5-m-deep soil cores across nine field sites in the eastern United States. The team characterized the impact of switchgrass on AMF communities, soil organic C, radiocarbon (^{14}C), root abundance, and a range of soil physical and chemical properties. AMF diversity decreases linearly below 40 cm depth. At most sites, deeply rooted switchgrass extended the habitat of AMF down the soil profile compared to the shallow-rooted controls (maximum AMF depths under switchgrass ranged from ~25 cm to 175 cm). By moving AMF down the soil profile, deep root systems can potentially extend the influence of AMF to impact subsoil C cycling and weathering processes.

Nuccio, E. E., et al. 2022. "HT-SIP: A Semi-Automated Stable Isotope Probing Pipeline Identifies Cross-Kingdom Interactions in the Hyphosphere of Arbuscular Mycorrhizal Fungi," *Microbiome* **10**, 199.

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Multiomics Pipelines and Approaches to Characterize Viral Impacts on Environmental Microbiomes

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Project Goals: The overarching goals of this project are to establish an analytical and experimental framework for comprehensive characterization of viral-driven alteration of microbial metabolisms in soil. The specific results presented here focus on the development of new tools and resources to help researchers "see" the viral signal in their data, and the benefits of pairing metagenomics and metatranscriptomics approaches to better characterize the potential impacts of viruses in microbiomes.

Metagenomics has emerged as a powerful approach to explore environmental viral diversity and identify the potential impacts of viruses in microbiomes, including in complex ecosystems such as soil. As the throughput, quality, and range of omics data expand, new methods and tools are needed to help researchers leverage the growing viromics toolkit and more thoroughly characterize uncultivated viruses beyond genome diversity. Here, researchers first outline how datasets, including paired metagenomes and metatranscriptomes, can help investigate viral activity in microbiomes. Specifically, in both longitudinal sampling of a mountainous soil and diurnal sampling of a Yellowstone hot spring microbial mat, only a limited fraction (~20 to 50%) of viruses identified via metagenomics were typically detected as transcriptionally active, and sample ordination based on metatransciptomic coverage provided a much stronger sample clustering consistent with ecological parameters compared to similar ordinations based on metagenomic coverage. This indicates that viral dynamics and potential impacts on a microbiome can be better understood by considering transcriptional activity in addition to detection in a metagenome. Next, the group presents Multi-choice Viromics Pipeline (MVP), an integrated workflow designed to enable researchers to run standard viromics analysis of metagenomes and/or metatranscriptomes in only a few easy steps. Integrating state-of-the-art tools, MVP enables nonexpert users to seamlessly process a set of metagenomes into heatmaps and ordinations based on viral signal, immediately providing a window into the viral diversity present in these data. MVP also automates a number of tasks, such as correcting quality estimation of provirus predictions, and provides summary statistics throughout the workflow to inform users on the overall viral content of their sample. Ultimately, the development of new viromics approaches, such as community-wide analysis of viral diversity through paired metagenomics and metatranscriptomics, along with the expansion of the viromics toolkit with both new tools and integrated user-friendly pipelines, will pave the way toward widespread adoption of these analyses and robust consideration of the role(s) of viruses in all microbiome studies.

Camargo, A. P., et al. 2023. "Identification of Mobile Genetic Elements with geNomad," *Nature Biotechnology* DOI:10.1038/s41587-023-01953-y.

Coclet, C., et al. 2023. "Virus Diversity and Activity is Driven by Snowmelt and Host Dynamics in a High-Altitude Watershed Soil Ecosystem," *Microbiome* **11**. DOI:10.1186/s40168-023-01666-z.

Roux, S., et al. 2023. "iPHoP: An Integrated Machine Learning Framework to Maximize Host Prediction for Metagenome-Derived Viruses of Archaea and Bacteria," *PLoS Biology* **21**(4). DOI:10.1371/journal.pbio.3002083.

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Other

Characterizing Mechanistic Roles of Viruses in Driving Biogeochemical Cycles in the Rhizosphere

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Project Goals: The information and tools generated from this project will address the need for the exploration of interkingdom interactions, specifically viruses, which has been highlighted as a priority by the Biological Systems Science Division strategic plan. The project seeks to develop a dynamic visualization tool that will rapidly allow for the identification of plant–microbe linkages. Through inter- and intracellular interrogation of viral-mediated signaling, regulation, and communication within plant–microbe interactions, the team aims to determine and predict how the soil virome affects ecological functions in soil and modulates global nutrient cycling.

Plant phenotypes are influenced by their microbiome, which consists of a dynamic consortium of bacteria that can provide benefits to the plant such as increased nutrient availability and stress resilience. There are well-studied examples of bacteria that have positive and negative effects on plants. There is also circumstantial evidence that viruses infecting these bacteria (bacteriophages) can alter their metabolism, as observed where phages are integrated into bacterial genomes (prophages). However, while viruses are ubiquitous, diverse, and the most abundant biological entities on the planet, their role in modulating plant-associated microbiomes remains poorly understood. The potential of viruses to impact global elemental cycles at massive scale is exemplified by the discovery of a "viral shunt" mechanism in marine ecosystems, where viral activity redirects nutrients and causes the release of up to three gigatons of carbon annually (Breitbart et al. 2018). A similar phenomenon has been described in soil systems, where viruses can influence nutrient availability and plant productivity (Wang et al. 2022).

Despite the clear importance of viruses in soil microbiomes, their role in regulating microbe–microbe and plant–microbe interactions in the rhizosphere (the microenvironment at the interface of roots and soil) is unknown. To understand these processes, it is necessary to study the properties of plants, bacteria, and viruses within the context of a multipartite functional system, establishing links between viruses and the ability of infected bacteria to colonize plant roots and influence plant phenotypes.

A recent Laboratory Directed Research and Development (LDRD) Lawrence Berkeley National Laboratory award has enabled the team to identify a viral–bacterial–plant tripartite system where prophages of the root-colonizing bacterium *Pseudomonas simiae* WCS417 were detected and experimentally determined to be active. The project also leveraged high-throughput mutagenesis (randomly barcoded transposon insertion sequencing; RB-TnSeq) to evaluate the potential for viral genes to modulate the colonization efficiency of their bacterial host. Two of these genes that cause reduced fitness in the rhizosphere when mutated are components of a latent bacteriophage and are present among two phage loci ranging in size from 15 to 65 kilobase pairs.

Using a loss of function approach, researchers generated green fluorescent protein–labeled phage gene deletion mutants to conduct experimental characterization studies such as competition tests, root colonization assays, and phenotypic comparative assessments. The team identified clear changes in metabolic profile between no bacteria controls and bacteria treatments from pilot *in vivo* targeted metabolomics experiments conducted in liquid growth media. These findings suggest the possibility that bacteriophages are involved in modulating the ability of bacteria to colonize plants. This approach therefore supports understanding and predicting how viruses may impact a given microbiome. However, extending understanding of these relationships more broadly across the rhizosphere is limited by the ability to connect the diversity of bacteria and prophages, as it relates to plants.

The project proposes to further understand the role of soil viruses in modulating plant-associated bacteria and to shed light on interkingdom signaling, resource sharing, and global nutrient cycling. By using an integrated computational and experimental design, the team seeks to understand how viruses modulate plant–microbe interactions, contribute to nutrient cycling, and work in response to dramatically altered water availability wrought by a changing climate.

Breitbart, M., et al. 2018. "Phage Puppet Masters of the Marine Microbial Realm," *Nature Microbiology* **3**, 754–66.

Wang, S., et al. 2022. "Experimental Evidence for the Impact of Phages on Mineralization of Soil-Derived Dissolved Organic Matter Under Different Temperature Regimes," *Science of the Total Environment* **846**, 157517.

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Microbial Treatments to Increase Carbon Sequestration in Biofuel Crop Systems

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Improving soil carbon sequestration in agricultural systems is critical for reaching net-zero carbon goals. It is estimated that 50% of soil carbon in agricultural lands has been lost, making them a natural sink for rapid carbon restoration. In these systems, plant carbon inputs to the soil, such as root exudates and liter, are rapidly metabolized by microbes back to carbon dioxide (CO_2). However, recent research shows that differences in soil microbial communities can produce three-fold differences in the amount of dissolved organic carbon, leading to a proportionate decrease in CO_2 release to the atmosphere. This combined with the fact that microorganisms can beneficially influence plant growth, carbon uptake, and root morphology, promoting deeper rooting, suggests that microbiome optimization either alone or combined with other plant treatments could be a solution for increasing soil carbon sequestration in agricultural systems without compromising crop yield. One of the main challenges for microbiome optimization is to develop and produce microbiomes that maintain their viability in natural environments.

To develop beneficial microbiomes that could maintain their viability in natural soils, researchers tested inoculating plant seeds with growth-promoting endophytic bacteria that live within the plant cells. The team hypothesized that, provided successful inoculation, this habitat would protect the beneficial microbes from the impacts of the complex existing microbiome in natural soils and help them maintain their beneficial effects on plant growth and carbon uptake. After developing the endophytic inocula, researchers tested their impacts on plant growth in the laboratory on *Camelina sativa* and sorghum, and on greenhouse gas emissions during growth in natural soils for *C. sativa* utilizing a soil core–based testbed with arid agricultural soil collected from a *C. sativa* field in the greenhouse. The group also compared the effects of endophytes on greenhouse gas emissions to the effects of inoculating the soil with nitrogen-fixing cyanobacteria.

The preliminary results show that, overall, inoculation with the endophytes slightly decreased CO_2 emissions from the plant–soil system for *C. sativa* but increased the nitrous oxide (N_2O) emissions or reduced N_2O soil sink at high water contents compared to uninoculated plants. Interestingly, the effects of inoculating the topsoil with nitrogen-fixing cyanobacteria on CO_2 and N_2O emissions was similar to having plants with or without endophyte inoculation. In both cases, the CO_2 and N_2O emissions increased above bare soil at midrange soil moisture contents (20 to 28%), and the natural soil N_2O sink at soil moisture contents close to saturation was removed. This suggests that plant growth–promoting endophytes can positively influence carbon sequestration in soil–plant systems, but the effects and their magnitude will depend on complex interactions between the plant–soil–microbiome systems and environmental conditions.