Systems Biology–Enabled Research for Microbial Production of Advanced Biofuels

Summary of Projects Awarded in 2014 Under Funding Opportunity Announcement DE-FOA-0001060

The U.S. Department of Energy's (DOE) Genomic Science program, managed within the Office of Biological and Environmental Research (BER), supports fundamental research to identify the foundational principles that drive biological systems. These principles govern translation of the genetic code into integrated networks of proteins, enzymes, regulatory elements, and metabolite pools underlying the functional processes of organisms. To address DOE's mission in sustainable bioenergy development, the Genomic Science program applies "omics"-driven tools of modern systems biology to challenges associated with microbial production of advanced biofuels.

Developing an increased understanding of how biological systems function and translating that knowledge to enhance the production capabilities of microbes and plants forms the basis of DOE's mission in sustainable bioenergy. To harness the microbial world's biosynthetic processing power for advanced biofuels production, an expanded set of platform organisms is needed with appropriate metabolic capabilities and stress tolerance characteristics. The DOE BER Genomic Science program supports research aimed at improving fundamental understanding of principles that govern the functional properties of bioenergy-relevant organisms at the genome scale. This knowledge will enable development of molecular genomics approaches and computational tools for the design, construction, and validation of improved biological components and systems. This highly interdisciplinary endeavor spans multiple fields in biology, systems biology, chemical and metabolic engineering, and computational biology.

Significant progress in the last decade has increased understanding of biological systems and the capabilities for manipulating them. These advances result largely from the tremendous technological leaps in developing molecular biology tools (e.g., genomic, metabolomic, and proteomic tools) to analyze and modify the functional properties of biological systems. Despite these advances, many fundamental gaps remain in understanding microbial metabolism and physiology related to the production of sustainable, efficient, and economically competitive biofuels derived from lignocellulosic plant biomass or from photosynthetic capture of carbon dioxide (CO_2).

The 2014 Funding Opportunity Announcement described herein specifically targets production of advanced biofuels, which in this context refers to biologically synthesized compounds with the potential to serve as energy-dense transportation fuels (e.g., diesel, gasoline, and aviation fuels) compatible with existing engines and fuel distribution infrastructures. Advanced biofuels production requires significant progress in the basic understanding of microbial metabolism and the conversion of photosynthetically derived carbon compounds (either via direct photosynthesis or acquired via breakdown of lignocellulosic plant biomass). Another goal is to determine how products can be efficiently shunted from central metabolism into complex products with associated rebalancing of organismal carbon allocations and redox potential.

BER solicited applications for systems biology–driven basic research in three areas of development focused on enabling advanced biofuels production:

- **Promising new model organisms relevant to biofuels production.** Proposed studies could include but are not limited to (1) advancing systems biology understanding and predictive modeling of specialist microbes or microbial consortia, (2) elucidating relevant regulatory and metabolic networks involved in product synthesis or environmental signal processing, (3) improving fundamental understanding of integrated function and compatibility of novel enzyme systems with direct applicability to lignocellulose breakdown or advanced biofuels production, and (4) developing genetic tools to facilitate study and manipulation of genetically intractable species.

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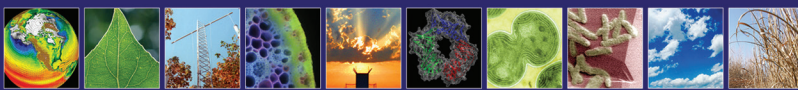
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- **Novel microbial functional capabilities and bio-synthetic pathways relevant to advanced biofuels production and strategies to overcome associated metabolic challenges resulting from pathway modification.** Proposed studies could include but are not limited to (1) development of robust and efficient pathways for advanced biofuels synthesis, (2) functional processes involved in deconstructing lignocellulosic plant material, (3) elucidation and modification of phenotypes involved in tolerance to stresses relevant to biofuels production, and (4) development of methods to overcome problems with recombinant expression of vital enzymes and pathways.

- **Novel analytical technologies or high-throughput screening approaches.** In conjunction with research addressing the two previously outlined topic areas, investigators may propose the development of analytical technologies facilitating characterization of relevant functional processes or high-throughput phenotyping of modified biofuel-producing strains. Only proposals for technology development tightly integrated with research addressing the two preceding topics will be considered.

2014 Awards

Systems Biology of Synthetic and Natural Lichens for Biofuels

- **Principal Investigator:** Michael Betenbaugh (Johns Hopkins University)
- **Collaborators:** Pam Silver (Harvard University), Karsten Zengler (University of California, San Diego), George Oyler (Johns Hopkins University), and Michael Guarnieri (National Renewable Energy Laboratory)

Project Goal and Summary: Construct symbiotic relationships between microbial phototrophs (cyanobacteria or algae) and heterotrophs (bacteria or yeast), enabling development of a coupled system for light-driven CO_2 fixation and high-efficiency synthesis of oils suitable for use as biofuels. Drawing inspiration from the natural symbiotic pairing of algae and fungi that form lichens, the investigators will examine specific factors that facilitate beneficial pairings between organisms and increase overall process efficiency. These pairings will focus on a phototroph engineered to secrete carbohydrates (*Synechococcus elongates*), first with a model bacterium (*Escherichia coli*) and later with an oleaginous yeast (*Yarrowia lipolytica*) that naturally accumulates oils. Transcriptomic and metabolomic approaches will facilitate construction of a genome-scale model to inform engineering efforts and enable fundamental insights into the development of consortial relationships among microbes. In the future, this type of relationship could enable effective division of labor in biofuels production, with specialization mitigating the metabolic overload observed in some engineered species that both photosynthesize and produce fuels.

Development of the Oleaginous Yeast *Rhodosporidium toruloides* as a New Model Organism for a Systems-Level Analysis of Lipid Productivity

- **Principal Investigator:** Rachel Brem (Buck Institute for Research on Aging)
- **Collaborators:** Jeffrey Skerker (University of California, Berkeley) and Adam Arkin (Lawrence Berkeley National Laboratory)

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

Systems Biology of *Rhodococcus opacus* to Enable Production of Fuels and Chemicals from Lignocellulose

- **Principal Investigator:** Gautam Dantas (Washington University, St. Louis)
- **Collaborators:** Marcus Foston and Tae Seok Moon (Washington University, St. Louis)

Project Goal and Summary: Develop *Rhodococcus opacus*, a soil bacterium capable of converting phenolic compounds to biofuel precursors, as a model microbe and potential platform for biofuels production. Phenolic compounds are released from lignin during biomass deconstruction and are problematic because of their potential toxicity to the microbes used for biofuels synthesis. By metabolizing these toxic compounds, *R. opacus* bypasses this problem and could increase biofuel production titers by utilizing lignin as a feedstock. Beginning with model phenolic substrates and eventually moving to thermochemically depolymerized lignin, the investigators will construct strains of *R. opacus* for increased tolerance to phenolics and increased biofuel precursor production. The evolved strains will then undergo a battery of omics testing to elucidate the mechanisms responsible for these desirable characteristics. Research results will advance systems biology understanding of genetic factors involved in resistance to lignin-derived phenolic toxins and could provide a promising new candidate platform organism for converting lignin compounds into biofuels.

A Systems Biology and Pond Culture-Based Understanding and Improvement of Metabolic Processes Related to Productivity in Diverse Microalgal Classes for Viable Biofuel Production

- **Principal Investigator:** Mark Hildebrand (University of California, San Diego)
- **Collaborators:** Juergen Polle (Brooklyn College of City University of New York) and Michael Huesemann (Pacific Northwest National Laboratory)

Project Goal and Summary: Investigate systems biology properties of several nonmodel algal species in the laboratory and under simulated outdoor conditions. This approach will allow investigators to focus their efforts on understanding regulatory and metabolic networks that impact growth rates and lipid yields in realistic biofuels production scenarios and enable potential improvements via targeted genetic modification. Investigators will work with four species representing the two major algal subgroups—the green chlorophytes

(*Scenedesmus obliquus* and a *Coelastrum* strain) and the stramenophile diatoms (*Thalassiosira pseudonana* and *Cyclotella cryptica*). This research will develop several algal species as model organisms and potential platforms for biofuels production, emphasizing the identification of specific genetic factors underlying performance in realistic biofuel production conditions.

Construction of a Robust Non-Oxidative Glycolysis in Model Organisms for n-Butanol Production

- **Principal Investigator:** James Liao (University of California, Los Angeles)

Project Goal and Summary: Construct and evolve model strains of bacteria (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*) to rely solely on an engineered pathway, non-oxidative glycolysis (NOG), for glucose utilization. The NOG pathway enables full retention of carbon from sugars when combined with reducing equivalents from formate or hydrogen, theoretically increasing carbon yield from 66% to 100%. Numerous genetic modifications are envisioned, including enzyme introduction in the NOG pathway and removal of essential glycolytic enzymes to force utilization of the engineered pathway. This development will be followed by selection for growth on glucose, as well as genomic and transcriptomic evaluation of the evolved strains to characterize their adaptions to NOG. The project also will explore direction of carbon flux into *n*-butanol, a potential advanced biofuel, in NOG-utilizing bacteria. Conversion of all carbon in sugar, aside from that required for cellular function, to next-generation biofuels could greatly improve the efficiency of microbial biofuels synthesis as well as illuminate the basic governing principles underlying fundamental rewiring of central carbon metabolism.

Ensemble Cell-Wide Kinetic Modeling of Anaerobic Organisms to Support Fuels and Chemicals Production

- **Principal Investigator:** Costas Maranas (Pennsylvania State University)
- **Collaborators:** James Liao (University of California, Los Angeles) and Greg Stephanopoulos (Massachusetts Institute of Technology)

Project Goal and Summary: Develop a systematic procedure to construct cell-wide kinetic models of two biofuel-relevant microbes through an ensemble modeling approach. This innovative modeling approach uses experimental transcriptomic, metabolomic, and

fluxomic data to limit possible metabolic pathways for carbon flux in model space to those most likely to represent actual physiology. This research focuses on two related microbes—*Clostridium thermocellum* and *Moorella thermoacetica*. Previously engineered for biofuels production, *C. thermocellum* is an efficient degrader of lignocellulosic biomass. *M. thermoacetica* is a homoacetogen being developed as a potential platform organism for converting synthesis gas ("syngas," a mixture of H_2, CO_2, and carbon monoxide produced during thermochemical conversion of biomass) to liquid biofuels. Research results will significantly advance the status of these two bioenergy-relevant microbes as model organisms and provide predictive modeling tools that will facilitate more sophisticated approaches to engineering microbes for improved biomass deconstruction and biofuels synthesis.

Systems-Level Study of a Novel Fast-Growing Cyanobacterial Strain for Next-Generation Biofuels Production

- **Principal Investigator:** Himadri Pakrasi (Washington University, St. Louis)
- **Collaborators:** Costas Maranas (Pennsylvania State University) and Yinjie Tang (Washington University, St. Louis)

Project Goal and Summary: Examine the systems biology properties of the cyanobacterium *Synechococcus elongatus* strain UTEX 2973 and develop modeling and bioengineering capabilities for this organism. Researchers recently demonstrated that this strain possesses the fastest growth rate of any known cyanobacterium. Despite this pronounced difference in growth rate from other more developed model strains of *Synechococcus*, genome sequencing has revealed a less than 1% difference in sequence in the 2973 strain, suggesting that this capability could be leveraged for more efficient biofuels synthesis and potentially engineered into other organisms. As such, development of this strain as a model organism and potential platform for light-driven conversion of CO_2 into biofuel compounds could have major impacts.

Quantitative Analysis of Metabolic Regulation by Integration of Metabolomics, Proteomics, and Fluxomics

- **Principal Investigator:** Joshua Rabinowitz (Princeton University)

Project Goal and Summary: Use an omics-driven systems biology approach to identify key points of integration between regulatory and metabolic networks in two microorganisms—the biofuel-producing yeast *Saccharomyces cerevisiae* and the cellulose-degrading bacterium *Clostridium celluloyticum*. Tight regulation of important metabolic pathways that control carbon flux continues to be a challenge for engineered biofuels production, and systematic elucidation of these control strategies is the first step to overriding this regulation. By analyzing data from metabolomics, proteomics, and fluxomics experiments, investigators will determine where the observed metabolic flux variations of reactions cannot be accounted for under standard models, identifying possible points of regulation and enabling use of a computational modeling approach to predict mediating elements. These predictions will be experimentally validated (1) *in vivo* by tuned modulation of expression of enzymes of interest, (2) *in vitro* by biochemical analysis of reaction kinetics displayed by enzymes in the presence of putative regulators, and (3) through systems-level genetic modification aimed at "unwiring" regulation in select cases. The resulting data will be used to refine systems-level metabolic and regulatory models for the two organisms, which will serve as valuable resources for both understanding their systems biology and guiding bioengineering strategies. This research will advance a sophisticated new technical approach for analyzing metabolic and regulatory networks and will generate new understanding of systems biology properties of two biofuel-relevant model organisms of broad value to the scientific community.

The Systems Biology of Protein Acetylation in Fuel-Producing Microorganisms

- **Principal Investigator:** Christopher Rao (University of Illinois, Urbana-Champaign)
- **Collaborator:** Alan Wolfe (Loyola University Chicago)

Project Goal and Summary: Determine how lysine acetylation alters production rates and yields in biofuel-producing strains of *Escherichia coli*. Multiple

studies have shown that metabolic enzymes are highly acetylated and that growth on different carbon sources changes their acetylation profile. This finding leads to the hypothesis that bacteria employ lysine acetylation as a global mechanism to regulate metabolism in response to their energy and redox status. The investigators will test the hypothesis that lysine acetylation plays a role in central metabolic pathways involved in the synthesis of biofuel compounds, leveraging previously engineered *E. coli* strains capable of synthesizing a variety of potential biofuel precursors. This research will address a key knowledge gap in global regulatory mechanisms (specifically at the translational level) used by bacteria to modulate large-scale cellular processes, focusing on the integration to regulatory and metabolic networks involved in biofuels synthesis.

Spatial Connectomics to Identify Agents Relevant to Lignocellulose Deconstruction in Fungi

- **Principal Investigator:** Jonathan Schilling (University of Minnesota)
- **Collaborators:** James M. Bradeen, Melania Figueora, and Jiwei Zhang (University of Minnesota) and Kenneth Hammel and Christopher Hunt (U.S. Department of Agriculture Forest Products Laboratory)

Project Goal and Summary: Examine the genetic basis of wood deconstruction in brown rot fungi, a subclass of wood-degrading fungi evolved from the more common white rot fungi. As compared to white rot fungi, brown rot species use a distinct and potentially more efficient mechanism to circumvent the lignin barrier during biomass breakdown. Investigators will use coupled omics techniques to spatially and sequentially map the expression of genes involved in wood deconstruction in fungal hyphae at the microbe-lignocellulose interface. This approach will employ an analytical technique developed in a prior DOE Early Career Research award that enables high-resolution transcriptomics and proteomic analysis of fungal hyphae in the active reaction zones in wood samples. This strategy will facilitate a highly focused comparison of expressed genes and secreted cellulolytic enzymes, resulting in development of a "connectome" co-localizing expression of implicated genes and proteins with specific degradative reactions in the wood. This work will advance overall understanding of mechanisms used by filamentous fungi to deconstruct lignocellulose and facilitate their potential application to production of next-generation biofuels.

A High-Throughput Pipeline for Mapping Inter-Species Interactions and Metabolic Synergy Relevant to Next-Generation Biofuel Production

- **Principal Investigator:** Daniel Segre (Boston University)
- **Collaborators:** Trent Northen (Lawrence Berkeley National Laboratory) and Christopher Marx (University of Idaho)

Project Goal and Summary: Examine interactions in a model microbial consortium relevant to consolidated bioprocessing and develop a computationally enabled pipeline for high-throughput omics analysis, predictive modeling, and targeted optimization of microbial consortia. The investigators have selected four model microorganisms, each with a different functional capability: (1) cellulose/hemicellulose degradation (*Streptomyces reticuli*), (2) lignin demethoxylation (*Methylobacterium extorquens*), (3) lignin degradation (*Streptomyces viridosporus*), and (4) biofuels synthesis (*Yarrowia lipolytica*). A stable, optimized consortium of these organisms theoretically would be capable of near-complete conversion of plant biomass into lipids suitable for biodiesel production. Research results will advance understanding and predictive modeling capabilities for community-scale microbial interactions and enable development of new consolidated bioprocessing strategies for biofuels synthesis.

Systems Biology Toward a Continuous Platform for Biofuels Production

- **Principal Investigator:** Janelle Thompson (Massachusetts Institute of Technology)
- **Collaborators:** Kristala Prather (Massachusetts Institute of Technology) and Michael Timko (Worcester Polytechnic Institute)

Project Goal and Summary: Examine systems biology properties of *Bacillus megaterium* strain SR7, a bacterium isolated from a deep subsurface salt dome capable of growth in the presence of super critical CO_2 ($scCO_2$). By developing this microbe as a potential biofuel production platform organism, investigators hope to enable development of continuous-flow bioreactors that are highly resistant to contamination and utilize $scCO_2$ chemistry for extraction of biofuel compounds. In addition to examining fundamental physiological properties of *B. megaterium* SR7, the team will engineer metabolic pathways for synthesizing medium-chain hydrocarbons and develop a bench-scale $scCO_2$ bioreactor system.

Research results will advance fundamental understanding of a newly discovered set of stress-tolerance characteristics highly relevant to DOE missions in bioenergy and environmental process understanding and enable development of a novel platform organism suitable for use in an innovative new biofuel-production process.

Unravel Lipid Accumulation Mechanism in Oleaginous Yeast through Single Cell Systems Biology Study

- **Principal Investigator:** Xiaoliang Xie (Harvard University)
- **Collaborator:** Shi-You Ding (National Renewable Energy Laboratory)

Project Goal and Summary: Better understand lipid accumulation in the oleaginous yeast *Rhodotorula glutinis* through development of single-cell analysis techniques. *R. glutinis* natively produces high levels of lipids and can accumulate them to a large fraction of its dry cell weight, making it a very promising organism for biofuels production. This research will focus on analyzing transcriptomic data from single cells correlated with quantitative measurements of lipid production *in vivo* through development of a stimulated Raman scattering microscopy (SRS-M)–enabled microfluidic flow sorter. The genes and transcription factors found to be potentially responsible for lipid accumulation will be verified through metabolic engineering strategies. This work will develop a novel technology for imaging and analysis of shifts in gene expression at the single-cell level, helping to advance this particular model organism for biofuels production and for systems biology research in the broader scientific community.

Next-Gen3: Sequencing, Modeling, and Advanced Biofuels

- **Principal Investigator:** Karsten Zengler (University of California, San Diego)
- **Collaborator:** Bernhard Palsson (University of California, San Diego)

Project Goal and Summary: Examine systems biology properties of the acetogenic bacterium *Acetobacterium ljungdahlii* and construct a genome-scale metabolic model. *A. ljungdahlii* is a potential chassis organism for biological conversion of synthesis gas ("syngas," a mixture of H_2, CO_2, and carbon monoxide produced during thermochemical conversion of biomass) to liquid biofuels. By advancing understanding of the nested genetic and metabolic networks of this organism and developing an *in silico* model of its biosystems, investigators aim to identify targets for metabolic engineering of strains capable of converting syngas to a variety of medium-chain alcohols. In addition to generating modified strains of *A. ljungdhalii* that potentially can be further developed as chassis organisms for syngas conversion, project results will facilitate new approaches to computationally driven design of biological systems and will significantly advance systems biology understanding of the homoacetogenesis, a form of chemolithotrophic metabolism with relevance to multiple DOE missions.

Further information on BER objectives in this area of research can be found on the Genomic Science program website at genomicscience.energy.gov/biofuels/. A list of the funded projects discussed in this document, as well as the Funding Opportunity Announcement that initiated them, also is available.

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