Systems Engineering of Rhodococcus opacus to Enable Production of Drop-in Fuels from Lignocellulose

Abstract (5000 characters, executive summary, keywords required)

Production of drop-in fuels from lignocellulose using *Rhodococcus opacus* PD630 (hereafter *R. opacus*) is a challenging goal. During the grant period we have pushed the field forward significantly in several areas of research. Towards the end goal of accelerating the adoption of *R. opacus* in biofuel production, during the grant period we have expanded the phenotypic characterization of *R. opacus* grown in single aromatic (model lignocellulosic) compounds or their mixtures, modeling the growth conditions in lignocellulosic biomass. Harnessing the power of adaptive evolution, we produced evolved *R. opacus* isolates with superior lignin valorization capabilities and identified differentially expressed genes and pathways after adaptation. We used next generation multi-omic techniques such as genomic, transcriptomic, and metabolomic analyses, to identify the catabolic pathways used by *R. opacus* to degrade aromatic compounds and funnel these degradation products into central metabolism, as well as the aromatic transport genes required for increased tolerance and utilization. Taking this information one step further, we identified endogenous transcription factors and regulatory mechanisms important for degradation of five model aromatic compounds. To accurately estimate *R. opacus* growth and consumption on model lignin compounds we pioneered the use of novel extraction procedures prior to GC-MS analysis. Alongside ^{13}C-metabolic flux analysis, we have elucidated the metabolic routes preferred by *Rhodococcus opacus* during aromatic compound degradation. Finally, we used in tandem lipidomics and high-resolution mass spectrometry to identify the modulation of mycolic acids and phospholipid membrane composition modification as a strategy for aromatic tolerance in *R. opacus*.

Being a non-model organism, *R. opacus* lacks the breadth of tools and technical foundation which drive biofuel research in more well-understood microbes such as *Escherichia coli.* To reduce this burden for use, we designed and produced new tools for genomic manipulation and engineering in *R. opacus*. These engineering breakthroughs support efficient genomic editing, enabling gene overexpression, repression, and genetic alteration. Using these tools, we have generated synthetically engineered strains with increased lipogenesis and growth, both positive traits required for increased lignin valorization. Optimizing engineered strains for biofuel production from lignocellulose requires extremely sophisticated synthetic rewiring of metabolism. To facilitate systems-level reorganization of metabolism in *R. opacus*, we created a genome-scale model that accurately predicts metabolic flux and growth rates on the aromatic compound phenol.

Lignin requires extensive pre-treatment before biological degradation by *R. opacus*. Towards an eventual goal of degrading real-world lignin, we developed new depolymerization processes to generate lignin breakdown products (LBP). We optimized LBP storage and composition analysis techniques, enabling accurate prediction of specific LBP compound integration into cell wall components. Overall, through the work funded by this grant we generated 20 manuscripts (17 published, 3 in review/preparation), methods for increased accuracy in metabolomics of aromatic compounds, multiple genetic tools for altering the *R. opacus* genome, genome scale models for predicting flux through metabolic pathways, as well as multi-omic data for community use. The work funded by this grant has increased the knowledge of aromatic degradation in bacteria and advanced our efforts to optimize *R. opacus* for lignin valorization.

Keywords: Lignocellulose, lignin valorization, synthetic biology, adaptive evolution, drop-in fuels, biofuels, *Rhodococcus opacus* PD630, genome-scale model, genomics, transcriptomics, metabolomics, lipidomics, aromatic degradation, ^{13}C-metabolic flux analysis

Technical Summary

Through this grant, we have designed, introduced, and standardized methods of biological engineering of *Rhodococcus opacus* PD630 (hereafter *R. opacus*) for valorizing lignocellulosic compounds for biofuel production. We optimized methods for measuring intracellular lipid, carbohydrate, and protein content, adapting a partial least squares (PLS) regression model using the Fourier-transformed infrared spectra of cells. The PLS model was shown to accurately predict carbohydrate content on a validation dataset without having to perform separate component extraction and analysis steps. For growth and consumption studies on lignin model compounds we developed methyl chloroformate derivatization and magnesium sulfate extraction procedures prior to GC-MS analysis.

We studied the effect of catalytic conditions on lignin breakdown products (LBP) yield and composition as well as the subsequent biological funneling by *R. opacus* and identified depolymerization conditions which wildtype *R. opacus* cannot grow on, related to the condition-dependent supernatant composition. We adaptively evolved *R. opacus* strains on aromatic compounds and compound mixtures to model the phenolic and aromatic derivatives generated in LBPs. Using growth, genomics, transcriptomics, and metabolomics data to characterize evolved *R. opacus* strains, we identified clusters of genes important for degrading aromatic compounds for assimilation into central metabolism via the beta-ketoadipate pathway. Employing ^{13}C-metabolic flux analysis we mapped the flux network of *R. opacus* and its adaptive mutants for catabolizing aromatic compounds. The MFA results delineate the functional carbon utilization pathways, and quantify the energy (NADPH, NADH, ATP) balance when cells use phenolic compounds. We then integrated the flux results with transcriptomics data to reveal metabolic regulation (i.e., genome-to-phenome mapping).

Utilizing evolved strains, we generated transcriptomic data from three evolved strains at concentrations of aromatic LBPs that are toxic to wild-type *R. opacus*, identifying shared differential expression of the beta-ketoadipate pathway and a novel putative operon likely involved in aromatic tolerance after adaptation. Using comparative transcriptomic analysis and systematic screening of regular-overexpressed strains on their lipid accumulation, we identified multiple transcription factors in *R. opacus* which significantly enhance lipid accumulation in nitrogen-replete conditions when overexpressed using either glucose or phenol as carbon source. Transcriptomic analysis revealed systematic changes caused by the overexpression of these regulators. Further genetic engineering allowed us to identify gene targets that are responsible for the enhanced lipid accumulation in *R. opacus*, creating 3 novel engineered strains with increased triacylglyceride production.

Our genome engineering tools have been successfully used for gene knockouts and knockdowns in *R. opacus*, providing insights into aromatic tolerance and utilization mechanisms. For example, gene knockout experiments confirmed the degradation routes of five aromatic compounds and identified relevant transcription factors and gene regulation mechanisms, including catabolic repression between aromatic compounds. Additionally, by knocking out three putative aromatic transporters identified from RNA-seq, we confirmed their role in lignin model compound tolerance and utilization.

The availability of genetic parts for tunable, high-activity gene expression is still limited. To address these shortcomings, we implemented inducible T7 RNA polymerase-based expression systems, demonstrating AND, NAND, and IMPLY logic gates. The increased expression enabled by the T7 systems was also applied toward improving our CRISPR interference (CRISPRi) platform for targeted gene repression (up to 82%), which was used to confirm several native aromatic pathways. Aromatic compounds are known to affect cellular membranes, but the role of lipid metabolism in aromatic tolerance of *R. opacus* is not well understood. We used a comprehensive lipidomic approach to understand aromatic tolerance mechanisms of *R. opacus* and identified >100 lipid species using high resolution mass spectrometry, finding that modulating the mycolic acid and phospholipid membrane composition may be one of *R. opacus*' strategies for aromatic tolerance. Finally, we created a genome-scale model for *R. opacus*: iGR1956. iGR1956 obtained an overall score of 93%. Growth rates predicted for growth on phenol were much more accurate than those predicted for growth on glucose.

In summary, the work funded by this grant has accelerated efforts to valorize lignin via bioproduction. Our major deliverables include *R. opacus* optimized protocols for metabolomics, a suite of

tools for genomic alteration and engineering, and the creation of over 35+ novel adapted and engineered strains with increased aromatic tolerance and other valuable qualities. Through the 20 manuscripts generated from this work, we describe discovered metabolic pathways, genes, and regulatory elements relevant to lignin valorization in *R. opacus*, highlighting specifically the beta-ketoadipate pathway is being vitally important to aromatic tolerance and degradation. Finally, by utilizing in-house created genome scale models and transcriptomic data, we have identified nodes within metabolic networks that can be tuned for increased lipogenesis, aromatic tolerance, and other lignin valorization enhancing characteristics. The tools and knowledge gained from this work provide concrete steps towards the development of *Rhodococcus opacus* PD630 as a chassis for bioproduction of drop-in biofuels and lignin valorization.

List of published manuscripts and patents

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