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Toward Lignin Valorization: Development of Rhodococcus opacus PD630 as a Chassis for Triacylglycerol (TAG) production from Recalcitrant Aromatic Feedstocks

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By

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Dedication

My last thesis was dedicated to my siblings.
It would be fair to make this one out to my parents.
After all, I love them very much, and they've been unceasingly supportive.
But no.

This one's for me.
I earned it.

Acknowledgements

My time at WashU has not been without its challenges, and there are many people who had a hand in getting me to this finish line. Perhaps most materially, my advisor, Dr. Tae Seok Moon, who took me on as a student despite a shaky first year, and who has kept me on for at least twice as long as we'd planned. It would be remiss to not also thank Drs. Marcus Foston and Yinjie Tang, whose agreement to serve on my committee is just the most visible piece of support they've provided me.

Former mentors Ray Henson and Drew DeLorenzo got me started in *Rhodococcus*, so it's fair to say they have been significant contributors. For the day-to-day support and discussions (some scientific, some less so), I would like to thank my past & present fellows in the Moon lab: Matt A, Austin, Jinjin, Yifeng, Kirsten, Matt L, Hyojeong, Anamika, Chenggang, Puneet, Franklin, Chenghao, Soo-Jung, Ryan, Junyang, Steven, Jeffrey, Tatenda, Abhijit, and all the rotation & undergraduate students who have cycled through. Elsewhere in the department, fellow grad students Ben & Claire, Jake, Garrett, Jeff, Pradeep, Albern, Anushka, and Oxford have provided diversion, companionship, and commiseration as required. I would also like to mention my collaborators, Winston over in the Dantas lab, the gents in the Foston lab, and the ladies of the department office; different sorts of collaboration, but all important.

In the before-times, Dr. Toivo Kallas was such a great advisor that I immediately signed on to do another graduate degree, so really this is his fault. My parents and siblings, of course, have encouraged me every step of the way; Mo, in particular, agreed to move down here with me so I wouldn't have to find a roommate. Finally, Rhodri may have no concept of what I do, but his emotional support has been essential.

Table of Contents

List of Tables

List of Figures

Abstract of the Thesis

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The advent of the industrial era was precipitated by the discovery of fossil fuels, and ushered in unprecedented changes for humanity included but not limited to the development of rapid transit and communications, improvements to food distribution and preservation, the mass production of goods, and a radical rearrangement of communities from relatively small enclaves to metropolises. With all the benefits, however, come considerable costs, especially to the global environment. Greenhouse gas emissions, built up over centuries of unregulated combustion, have precipitated a rate of global temperature change unparalleled in the 4.5 billion-year history of this planet. In order to preserve life on Earth, emissions must be dramatically reduced – first halved by 2030, and then diminished to a net-zero by 2050. One method to make these cuts is to explore renewable sources of energy and chemicals. One viable petroleum-alternative raw material is lignocellulosic biomass, the most abundant biopolymer in the world. Pretreatment of lignocellulose for depolymerization generates a range of compounds, including fermentable sugars which can be readily converted into alcohols for biofuel applications, and the recalcitrant aromatic portion, liginin. Due to costs associated with depolymerizing lignin and separating out the resulting mixture of heterogeneous, aromatic compounds, the lignin fraction is generally

separated from the sugar-based cellulose and hemicellulose fractions and burned to supplement energy requirements of the biorefinery. Valorizing lignin into value-added compounds, however, not only helps to reduce greenhouse gas emissions, it also is a key factor in improving the economic viability of a biorefinery.

One of the more promising avenues for lignin valorization being explored is its use as a substrate for bioproduction of commodity chemicals by microbial cell factories. A confounding factor in this method is identifying microbes with the capability to tolerate and consume a wide range of toxic aromatic compounds, combined with the metabolic basis to become a bioproduction chassis. *Rhodococcus opacus* PD630 (hereafter PD630) is a Gram-positive, non-model soil bacterium isolated from soil outside a German gasworks plant and enriched on phenyldecane. Among its desirable traits are a diverse range of compatible substrates ranging from sugars to lignin-derived aromatics, the ability to accumulate more than half its dry cell weight in triacylglycerols (TAGs, a precursor for biofuels and other chemicals), a rapidly-developing genetic toolbox, and moderately fast growth. This thesis examines the development of PD630 as a platform for lignin valorization, focusing in particular on conditions promoting TAG accumulation. To this end, the research herein presents a method for mining transcriptomic datasets for regulatory genes which are part of the response network for particular environmental conditions. In brief, a regulatory element is identified which supports nitrogen-independent accumulation of TAG, particularly when phenol is present in the media. As TAG production is largely upregulated in response to nitrogen starvation, and nitrogen stress inhibits aromatic tolerance in PD630, these findings represent progress toward optimizing PD630 as a biocatalyst for lignin valorization. Additionally, they elucidate some of the network rearrangements

necessary to support PD630 switching from a growth phenotype to TAG accumulation in a nitrogen-depleted environment.

Chapter 1: Introduction

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Abstract

The current extraction and use of fossil fuels has been linked to extensive negative health and environmental outcomes. Lignocellulosic biomass-derived biofuels and bio-products are being actively considered as renewable alternatives to the fuels, chemicals, and materials produced from fossil fuels. A major challenge limiting large-scale, economic deployment of second-generation biorefineries is the insufficient product yield, diversity, and value that current conversion technologies can extract from lignocellulose, in particular from the under-utilized lignin fraction. *Rhodococcus opacus* PD630 is an oleaginous gram-positive bacterium with innate catabolic pathways and tolerance mechanisms for the inhibitory aromatic compounds found in depolymerized lignin, as well as native or engineered pathways for hexose and pentose sugars found in the carbohydrate fractions of biomass. As a result, *R. opacus* holds potential as a biological chassis for the conversion of lignocellulosic biomass into biodiesel precursors and other value-added products. This review begins by examining the important role that lignin utilization will play in the future of biorefineries and by providing a concise survey of the current lignin conversion technologies. The genetic machinery and capabilities of *R. opacus* that allow the bacterium to tolerate and metabolize aromatic compounds and depolymerized lignin are also discussed, along with a synopsis of the genetic toolbox and synthetic biology methods now available for engineering this organism. Finally, we summarize the different feedstocks that *R. opacus* has been demonstrated to consume, and the high-value products that it has been shown to

produce. Engineered *R. opacus* will enable lignin valorization over the coming years, leading to cost-effective conversion of lignocellulose into fuels, chemicals, and materials.

Introduction

In comparison to estimated pre-industrial levels (circa 1700 CE), the current global atmospheric CO_2 concentration has increased over 100 parts per million (ppm) and is now stably maintained over 400 ppm, with three-quarters of that change occurring since 1960 due to fossil fuel emissions (1). This increase in the CO_2 concentration has already contributed to a small, but significant, rise in global average temperatures, and will lead to even greater increases in the future. Climate change can lead to decreasing crop yields and seed quality (1), facilitate sea level rise, promote destructive extreme weather events, and cause spikes in energy usage as increasingly frequent severe weather can cause unscheduled shutdown/startup cycles (2). Addressing greenhouse gas-driven climate change will require a complex, multi-tiered approach towards a more carbon-neutral world, including a greater usage of biofuels in the transportation industry and more sustainable chemical and material synthesis.

Biofuels or bio-products, derived from biological sources (i.e., biomass) rather than petroleum, are not a new concept. The inventor of the diesel engine advocated for farmers generating their own vegetable-oil fuel in areas lacking a consistent source of petroleum (3). First-generation biofuels and bio-products are derived from food crops like corn, soy, palm, and sugarcane (3-6). While these biofuels and bio-products have the potential to mitigate CO_2 emissions associated with fossil fuels (3, 5, 6), they are economically and environmentally problematic: energy uses related to irrigation, fertilizer production, cultivation, and transportation are significant, and global demand for food outweighs the supply of arable land (4, 6). Other sources of biomass, like lignocellulose, are readily available as by-products of the agriculture and

forestry industries. Moreover, the dedicated cultivation of properly selected and/or engineered species as sources of lignocellulose can be achieved in a wider distribution of climate and soil conditions with reduced water and fertilizer requirements compared to first-generation sources (7). However, lignocellulose does require more complex processing to produce a second-generation biofuel or bio-product. The structural component of lignocellulose, lignin, provides a particular challenge as it is a complex aromatic macromolecule that evolved to resist degradation (8). Complete utilization and upgrading of lignin is critical for economic viability of second-generation biorefineries.

The processing of lignocellulosic biomass can be split up into two steps: (1) depolymerization in which the polymers within lignocellulose (e.g., cellulose, hemicellulose, and lignin) undergo cleavage reaction producing their respective subunits; and (2) upgrading in which these subunits are converted into a value-added product. Both steps of biomass processing can be achieved through diverse biological, thermochemical, and catalytic processes. However, the generation of a single value-added product requires upgrading followed by extensive chemical separations, or the use of biological catalysts (i.e., a microbe) that can funnel and convert many different lignocellulose-derived substrates into a single product. *Rhodococcus opacus* PD630 (hereafter *R. opacus*) has been identified as a potential biological chassis for the funneling and conversion of lignocellulose-derived substrates to lipids, a biofuel precursor. *R. opacus* has a natural tolerance to toxic aromatic compounds found in the lignin fraction of lignocellulose, an ability to increase this tolerance through adaption, and numerous catabolic pathways for consumption of both carbohydrates and aromatics, making it an ideal candidate to address the challenges of biomass conversion (9). *R. opacus* has also shown the ability to accumulate up to ~80% of its cell dry weight in lipids, such as triacylglycerols (TAGs), under certain growth

conditions. These lipids can then be converted to biodiesel via a transesterification reaction (10, 11). In this review, we focus on discussing the important role of lignin valorization in regard to the viability of second-generation biorefineries, summarize different lignocellulose depolymerization methods, and examine *R. opacus*' potential for the conversion of biomass breakdown products into diverse fuels and chemicals.

Why bio-products?

Industrial oil drilling began in the mid-19th century, and in 2015 there was an assessed 35.2 billion barrels of proven oil reserves in the United States, with an estimated 3.4 billion barrels produced domestically that year (12). While predictions of "peak oil" made over the past two decades have been overly pessimistic, it is not unreasonable to predict that recovery of global oil reserves will, at some point, become economically unfeasible (13). Thus, an alternative renewable source of energy and substitutes for products derived from petroleum will be required in the future. Lignocellulosic biomass represents one of the few sources of renewable carbon, while renewable energy (e.g., electricity) can be generated from other renewable energy sources such as wind and solar energy. Biorefineries can convert biomass into a range of products by employing integrated catalytic, thermochemical, and biological conversion processes that efficiently utilize the carbon and energy stored in that biomass (14, 15). A future that does not rely on fossil resources will involve renewable electrical production paired with the generation of biomass-derived products.

The last two decades of research in carbohydrate conversion techniques have witnessed successful biofuel and biochemical production, but the conversion of the lignin fraction of biomass has been less explored (16). Federal regulation and clean energy initiatives are targeting production rates of 79 billion liters per year of second-generation biofuels by 2022, and the quantity of lignin remaining after the sugar fraction is fermented to reach that target could be as high as 62 million

dry tons annually (17-19). Increasing lignin utilization would not only help offset the environmental impact of biomass refinement, but also drastically increase the economic feasibility of the biorefinery. Second generation biofuels and value-added bio-products derived from biomass represent versatile end products, but the future commercial viability of biorefinery products depends on efficient use of both the carbohydrate and lignin substrates (20).

What is lignin?

Lignin is a complex and heterogeneous macromolecule composed of cross-linked aromatic monomers and imparts a rigid or "woody" characteristic of plants that helps provide structural support and limit degradation of polysaccharides. The molecular structure of lignin polymers is primarily derived from *p*-coumaryl, coniferyl, and sinapyl alcohols and corresponds to *p*-hydroxyphenyl, guaiacyl, and syringyl monolignol units, respectively; however, there are a variety of other units which occur less frequently (8, 21). The composition and relative abundance of each type of monolignol varies species-to-species, genotype-to-genotype, across tissue types, between cell wall layers, across different development stages, and as a function of environmental factors (22). The monolignols form several inter-monomer linkages, most commonly aryl ether bonds (e.g., β-O-4, α-O-4, and 4-O-5) (23). The extremely diverse and variable molecular structure of lignin makes commercial degradation difficult and widespread utilization of lignin challenging; however, effective utilization of lignin is necessary for lignocellulose conversion profitability (24).

Thermochemical and catalytic conversion of lignin

Research has been conducted to develop conversion technologies that deconstruct lignin, in particular lignin generated as a by-product of papermaking and biomass-derived carbohydrate fermentation, for the production of renewable fuels, chemicals, and materials (25). Lignin's inherent recalcitrance toward deconstruction makes it difficult to depolymerize for industrial

purposes (19, 26). Transforming lignin into higher value products is further complicated by its structural diversity and the high propensity of its intermediates to engage in secondary reactions. An identical lignin conversion process can generate different distributions of compounds depending on the chemical and molecular structure of the lignin feedstock. Additionally, each type of conversion technology has numerous processing conditions that determine the product phase (i.e., solid, liquid, or gas), composition, and application, as well as other conversion performance metrics (e.g. product yield, productivity, selectivity, and composition). For example, a liquid fuel product can easily be derived from lignin using pyrolysis, which exposes the lignin to high temperatures in the absence of oxygen (27). However, the chemical composition of this lignin-derived pyrolysis oil has such a wide distribution of compounds that it has little to no utility for chemical production (28).

Thermochemical and catalytic conversion technologies for lignin valorization primarily include pyrolysis, hydrothermal liquefaction (HTL), gasification, oxidative cracking, hydrogenolysis, and solvolysis (Table 1) (25, 29, 30). These lignin conversion technologies generate gas, liquid, and/or solid breakdown products through numerous complex reactions. Due to differing process conditions defining these conversion technologies, certain reaction pathways are favored, which alters the yield and composition of the breakdown products. Catalytic technologies (i.e., oxidative cracking, hydrogenolysis, and solvolysis), which have been reviewed in detail by Zakzeski et al, provide a promising avenue to convert lignin selectively into its constituent monomers or monomer derivatives (30). Aromatic carbon-oxygen bonds in aryl ether inter-monomer linkages, which comprise 50-60% of the inter-monomer linkages of lignin (31), represent a potential macromolecular “weak” point that could prove an effective target for selective depolymerization. In this case, a catalyst not only facilitates cleavage of specific bonds along the

lignin chains but also allows the cleavage to occur with a lower energy input, reducing the occurrence of secondary reactions with higher activation energies. The resulting product mixture would therefore have a much narrower distribution of aromatic compounds, which may be more amenable to cost-effective chemical separation and/or downstream upgrading.

Table 1-1. Summary of thermochemical and catalytic technologies for lignin conversion (25).

Technology	Main Product	Product Application	Process Notes
Gasification	Syngas (gas)	Production of energy, hydrogen & methanol (methanol synthesis); alkanes (Fischer-Tropsch); isobutane (isosynthesis); ethanol (fermentation & catalysts); aldehydes & alcohols (oxosynthesis)	Performed under high temperatures (>700°C); can involve the addition of water & catalyst
Fast pyrolysis / Hydrothermal liquefaction (HTL)	Bio-oil (liquid)	Production of energy and various liquid fuels (e.g., bio-gasoline) by catalytic upgrading	Performed at 250 to 700°C; can involve the addition of water (HTL), hydrogen (hydropyrolsis) & catalyst (catalytic pyrolysis)
Torrefaction / Slow Pyrolysis	Bio-char (solid)	Used as a more-optimized solid fuel for combustion	Performed at 200 to 350°C
Solvolysis	Soluble lignin fragments (liquid)	Phenolics & alkyl-phenolics	Two main categories: (A) acid- & base-catalyzed depolymerization (B) supercritical solvent depolymerization
Hydrogenolysis	Soluble lignin fragments (liquid)	Phenolics & alkyl-phenolics	Hydrogen donor (e.g., hydrogen gas, alcohol, or acid) & a catalyst can be used to cleave linkages
Oxidative cracking	Soluble lignin fragments (liquid)	Aromatic aldehyde, ketones & carboxylic acids	Linkages in lignin can be cleaved by an oxidant (e.g., air & hydrogen peroxide) & a catalyst

Biological and hybrid conversion of lignin

Although catalysts can provide a route toward selective lignin depolymerization chemistry, thermo-catalytic processing of lignin often results in a product mixture that still requires extensive chemical separations. Additionally, lignin depolymerization products are generally limited to aromatic- and phenolic-derivatives. There has been significant research studying the application of enzymes and various microorganisms as a more selective and facile method of lignin

depolymerization, as discussed in a recent report (32). In general, biological systems require mild conditions that avoid costs associated with the use of high temperatures and high pressures. However, only a few bacteria (e.g., *Streptomyces* spp., *Rhodococcus* spp., and *Nocardia* spp.) and brown/white-rot fungi (33, 34) have an ability to depolymerize lignin, and their lignin depolymerization rate is too low to be useful on an industrial scale (26, 35).

To overcome these challenges, researchers have adopted a hybrid conversion approach which combines the best attributes of thermo-catalytic and biological conversion technologies (36). In a hybrid conversion approach, a thermo-catalytic conversion process with advantageous reaction kinetics and conversion is applied for the initial lignin depolymerization. Downstream, microbial conversion and funneling of the depolymerized lignin breakdown products (LBPs) to a value-added product then occurs with advantageous selectivity (37). There are numerous aromatic catabolic pathways in various microbes which can be harnessed into a “biological funnel” by converting the heterogeneous substrates generated during depolymerization into common metabolic intermediates (e.g., protocatechuate and catechol) (38). These intermediates undergo further conversion to central metabolites (e.g., acetyl-CoA) that can be utilized to produce target compounds at a high selectivity.

Hybrid conversion technologies have been implemented, but they have almost exclusively focused on sugar utilization (39, 40). For example, cellulosic technologies can consist of thermochemical polysaccharide depolymerization (e.g., acid hydrolysis (41) or production of pyrolytic sugars (42)) and biological conversion of the resulting monosaccharides into ethanol or other products (39). Recent work has begun to shift the focus from sugar fermentation to lignin utilization, with most research concentrating on using lignin model compounds to characterize aromatic degradation pathways (43-45) and bioconversion abilities (46, 47). Demonstrations of an

integrated thermochemical process with an aromatic-metabolizing microbial catalyst using pretreatment liquors have been performed, but these feedstocks frequently contain only a portion of the original lignin content, as the pretreatment process has been optimized for sugar release via enzymatic hydrolysis rather than maximizing lignin conversion (38, 48, 49). For an effective lignin hybrid conversion process, the upstream thermochemical or catalytic depolymerization process must meet the following requirements: (1) production of aqueous soluble LBPs; (2) optimization of lignin conversion for yield and selectivity toward the preferred substrates for microbial growth and utilization; (3) minimal generation of inhibitor compounds; and (4) a process configuration and condition that is compatible with an economical, sustainable, and large-scale design. To this end, multiple lignocellulosic biomass pretreatment techniques have been tested, demonstrating the potential of hybrid conversion processes (48-52).

Why *Rhodococcus opacus* PD630 for hybrid conversion of lignin?

Rhodococcus opacus PD630 has been identified as a candidate biological catalyst for the conversion of both the carbohydrate and lignin fractions of lignocellulose into valuable products. *R. opacus* was originally isolated from soil collected near a gas works plant by enrichment on phenyldecane as a sole carbon source (11). *R. opacus* possesses extensive catabolic pathways for both sugars and aromatics and can tolerate inhibitory compounds found in depolymerized biomass (e.g., phenolics and furfural) (53). The ability to metabolize aromatic compounds is shared by a number of microorganisms and is likely a common evolutionary trait due to the prevalence of lignin in natural environments. Many of the aromatic compounds that *R. opacus* is known to metabolize can be found in LBPs (9, 54-58). In addition to lignin model compounds (e.g., 4-hydroxybenzoate, benzoate, phenol, vanillate, guaiacol, and trans-*p*-coumaric acid), *R. opacus* has been shown to degrade depolymerized kraft lignin (59), alkali-treated corn stover (60, 61), alkali-

treated poplar wood (34), and switchgrass pyrolysis oil (52). Through adaptive evolution, *R. opacus* has been further evolved to more efficiently degrade phenol, syringaldehyde, and aromatic mixtures (9, 53, 54). *R. opacus* has also been engineered using exogenous genes expressed on plasmids to degrade cellulose, arabinose, and xylose (62-64). It is therefore, through native or engineered means, able to tolerate and utilize a variety of typically toxic lignin-derived compounds, in addition to sugars.

Unlike most bacteria that store carbon as polyhydroxyalkanoic acids (PHAs), *R. opacus* stores carbon as energy-rich triacylglycerols (TAGs) (58, 65). Acetyl-CoA is the product of diverse catabolic pathways in *R. opacus*, including glycolysis, the Entner–Doudoroff pathway, and aromatic degradation pathways (e.g., β-ketoadipate pathway), and it is a key precursor in TAG biosynthesis. Under nitrogen limitation, the non-limiting essential nutrient (i.e., carbon) is stored as TAGs in *R. opacus*, accumulating up to ~80% of its cellular dry weight when cultured on gluconate (11, 66). On aromatic compounds, lipid production is reduced, but it can still reach up to 44% of cellular dry weight in TAGs under nitrogen-limiting conditions (9). *R. opacus* can also synthesize branched-chain and odd-numbered fatty acids that are necessary for next generation biofuels. Shifting lipid storage in *R. opacus* to these compounds would make it an even more valuable production strain (67).

Other organisms have been proposed for lignin conversion, but *R. opacus* has demonstrated higher or equal rates of aromatic degradation and tolerance compared to other species. For example, a phenol-adapted *Pseudomonas putida* strain had a maximum phenol degradation rate of ~12 mg phenol/L/hr when grown at its maximum tolerated phenol concentration of 1 g/L (68). *Bacillus brevis* previously claimed the highest phenol tolerance and utilization when cultures grew at concentrations up to 1.75 g/L phenol, and demonstrated a maximum degradation rate of ~20

mg/L/hr (69). Adapted *R. opacus* strains were able to grow at 2 g/L phenol, and demonstrated a maximum degradation rates of ~21-22 mg/L/hr (54).

In summary, *R. opacus* is an ideal candidate for the hybrid approach of lignocellulose utilization because of its high tolerance to aromatic compounds, its capacity to utilize a wide variety of substrates (both carbohydrates and lignin), and its ability to accumulate lipids. These traits are uniquely-suited to handling the aromatic mixtures produced by lignin depolymerization processes and metabolizing them into valuable compounds. Additionally, *R. opacus* is amenable to adaptive evolution to improve the tolerance and growth rate on aromatic and lignin substrates (9, 53, 54). These natural characteristics, along with a growing toolbox of genetic tools, make *R. opacus* an ideal organism for lignin valorization (70).

Genetic and metabolic characteristics of *R. opacus*

Aromatic degradation in *R. opacus* is facilitated by a high-flux β-ketoadipate pathway that produces acetyl-CoA (Figure 1-1) (71). As acetyl-CoA is the precursor molecule for many biochemicals, *R. opacus* is thus well suited for chemical production based on an aromatic feedstock. Additionally, glucose metabolism in *R. opacus* exclusively utilizes the Entner-Doudoroff pathway, enabling simultaneous utilization of phenol and glucose. This lack of catabolite repression means that *R. opacus* can effectively use both the carbohydrate and lignin fractions of lignocellulosic biomass with reduced fermentation times and increased productivities. While sugar and phenol metabolisms are independent, *R. opacus* degrades aromatic compounds in a preferential order (9, 72). It is unclear what is driving this preferential consumption, but it may result from variations in enzyme and transporter activities, or transcriptional-level regulation.

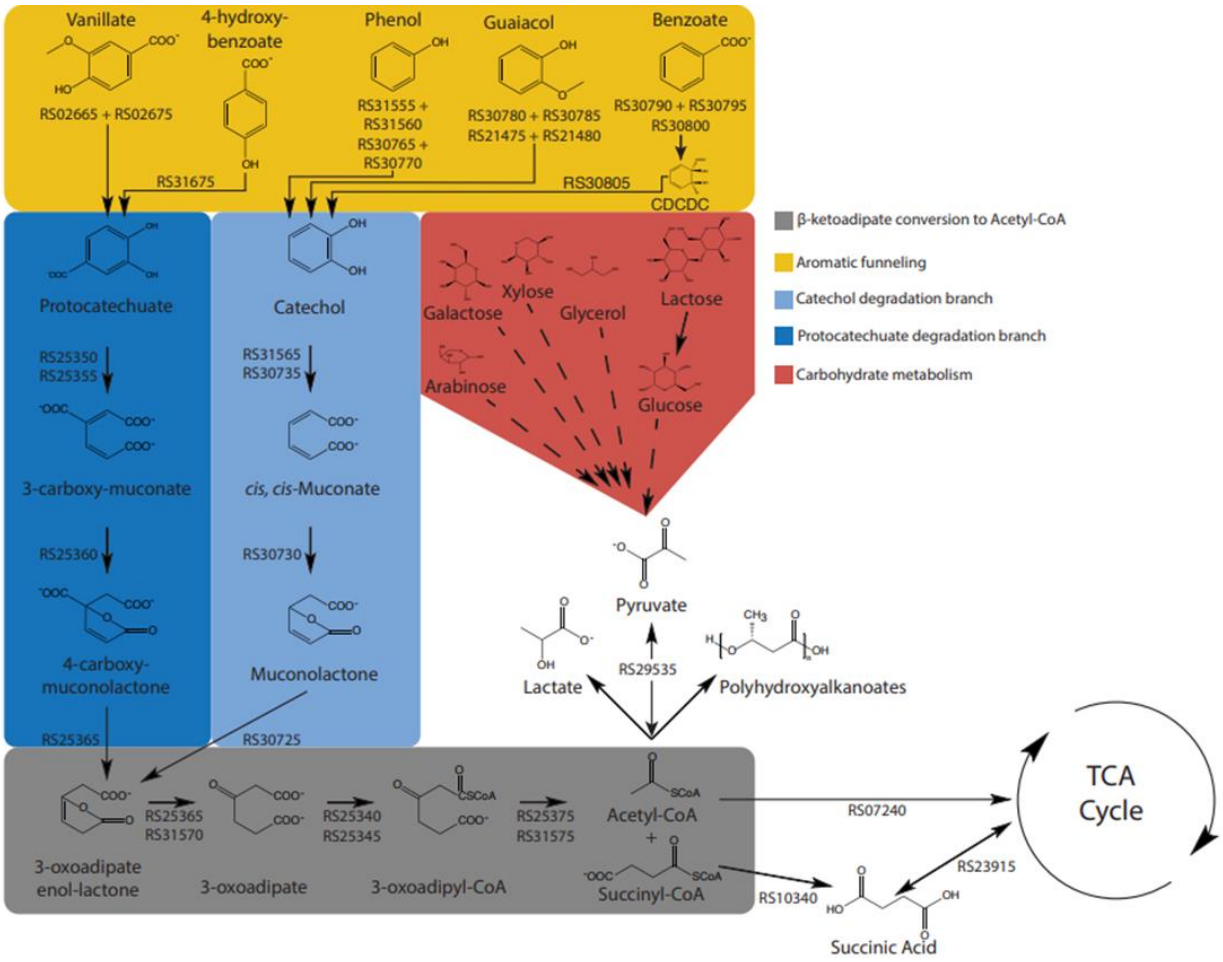

Figure 1-1. Aromatic degradation and carbon metabolism in R. opacus. R. opacus genes involved in reactions are listed. Dashed arrows represent multiple intermediate steps not shown. Xylose and arabinose consumptions occur via engineered pathways.

Aromatic compounds entering the cell generally first undergo preliminary degradation to either protocatechuate or catechol before being metabolized through the β-ketoadipate pathway. Mechanistically, this import into the cell and pre-processing occurs via specialized aromatic transporters and funneling enzymes, which have been identified using transcriptomics and proteomics (9, 34, 54). Henson et al. identified three aromatic-associated transporters: one specific to phenol, one associated with both phenol and vanillate, and a promiscuous transporter associated with phenol, vanillate, benzoate, and guaiacol (9). Several funneling enzymes have also been

identified, including those which convert vanillate and 4-hydroxybenzoate to protocatechuate, as well as those which convert phenol, guaiacol, and benzoate to catechol (9).

Advantageous mutations and transcriptional changes have been identified in *R. opacus* that could be future targets for additional growth optimization through forward engineering. Genes for enzymes involved in oxidation-reduction reactions underwent changes in multiple strains adapted for improved growth on one or more phenolic compounds, including cytochrome ubiquinol oxidase subunit I and superoxide dismutase (9, 54). The fact that functionally equivalent mutations occurred in multiple aromatic-adapted strains suggests their link to improved aromatic tolerance and utilization. For example, decreasing the activity of superoxide dismutase, as demonstrated in these mutated strains, may allow the cells to increase oxidizing equivalents, which are necessary to degrade highly-reduced aromatic rings. Transcriptomic analysis of adapted strains also identified increased expression of aromatic transporters, which correlated with increased phenolic tolerance and utilization. These and other changes identified in the genome and transcriptome of adaptively-evolved strains could be replicated in a rationally-engineered strain to fine-tune its growth on and tolerance to aromatic-rich substrates.

Additional potential targets for strain optimization in *R. opacus* are its nine endogenous plasmids (2 circular and 7 linear plasmids; a combined total of 0.79 Mbp). These plasmids have been posited to act as a hyper-recombinational gene storage strategy in which infrequently-used catabolic genes are stored on plasmids as a failsafe against rarely-encountered compounds (e.g., nitrophenolates and polycyclic and/or halogenated aromatics) which may be present in the environment (73-78). If genes located on a plasmid are found to be regularly useful, they can undergo recombination with the 8.38 Mbp circular chromosome and become permanent components of the genome. This strategy has been observed in strains of related *Actinomycetales*,

where stored genes provide the catabolic versatility to degrade a larger array of organic compounds (79, 80). Additionally, genes located on plasmids, particularly if they are duplicates not subject to evolutionary conservation, can collect mutations more rapidly than those genes in the chromosome, allowing for improved adaptive capacity. For potential industrial use with a relatively well-defined and consistent feedstock, tailored strains of *R. opacus* may benefit from the selective removal of some of these plasmids, as previously adapted strains that exhibited improved growth profiles on phenolic compounds underwent large deletions or complete loss of plasmids 1 (0.17 Mbp) and 2 (0.098 Mbp) (9). Plasmid removal under selective pressure may be driven by a reduced metabolic burden. Strategically, intentional plasmid curing would best be employed when cells are cultured on a defined range of carbon sources, where trading catabolic potential for improved growth rate is an acceptable risk.

Tool and technique development for R. opacus engineering

As *R. opacus* is a non-model bacterium, the available genetic tools and techniques for engineering this organism were relatively sparse until recently. The genetic toolbox available for *R. opacus* has expanded to include a reference genome (58, 65), plasmid backbones for gene overexpression, and promoters for tunable gene expression (Table 2). Furthermore, methods for performing gene knock-outs and knock-ins, modulating and quantifying gene expression, and extracting intracellular products via viral lysis have all been demonstrated. We summarize the most prominent genetic tools and techniques developed for several *R. opacus* strains.

A common element used for gene overexpression is the replicating plasmid backbone. As previously mentioned, *R. opacus* PD630 has 9 endogenous plasmids, which range in size from 37 to 172 Kbp (19). These endogenous plasmids have 1 to 5 copies per chromosome, with plasmid 8 (5 copies per chromosome) having a confirmed neutral site for stable integration of heterologous

DNA constructs (73). Additionally, there are a number of heterologous plasmids isolated from other *Actinomyces* spp. that have been demonstrated to replicate stably in *R. opacus* (Table 2). While they have several names, these plasmid backbones can be grouped into five primary categories. The pAL5000-based plasmid group, consisting of short and long variants, is derived from *Mycobacteria* spp. and has been demonstrated to have 3 to 11 copies per chromosome, dependent on the variant (73, 81, 82). The pNG2, pGA1, and pSR1 plasmid groups are all ancestrally related and derived from cryptic *Corynebacterium* spp. plasmids that replicate through rolling-circle amplification (83-87). A BioBrick-compatible version of pSR1 (pSRKBB) has recently been developed for easy cloning (88). The pAL5000 and pNG2 backbones have been demonstrated to be compatible, allowing co-maintenance of two heterologous plasmids (73). Finally, pB264, which is derived from an endogenous *Rhodococcus sp.* B264 plasmid, has ~8 copies per chromosome and is easily curable from the cell once antibiotic selection pressure is removed (73, 89). To ensure that heterologous plasmids are stably maintained within a cell, selection is required, most frequently in the form of an antibiotic resistance marker. Several of these markers have been demonstrated and optimized for selection in *R. opacus* (Table 2), though there may be room for refinement with regards to stable maintenance concentrations (88). A recent study employed single-cell fluorescence to demonstrate that the concentration of kanamycin sufficient for selection was not the ideal concentration for plasmid function maintenance. The commonly-used concentration of 50 µg/mL led to a bimodal population of cell fluorescence, with fewer than half of the cells demonstrating fluorescent reporter expression when analyzed via flow cytometry (88). Increasing the concentration of kanamycin to 250 µg/mL led to a majority of cells expressing the fluorescent reporter.

Table 1-2. List of genetic parts demonstrated in *R. opacus*, including plasmid backbones, selection markers, promoters, and recombination-related parts.

Part Type	Name	Properties/Notes	Source
Plasmid backbones	pAL5000 (short)	Other names: pXYLA and pNV18; ~11 copies per chromosome	(62, 73, 90-94)
	pAL5000 (long)	Other names: pJAM2 and pJEM; ~ 3 copies per chromosome	(63, 73, 90, 93)
	pNG2	Derived from *Corynebeacterium* spp.; ~10 copies per chromosome	(82, 84)
	pGA1	Derived from *Corynebeacterium* spp.	(63, 87, 95, 96)
	pSR1	Derived from *Corynebeacterium* spp.	(85, 86, 88)
	pB264	Derived from *Rhodococcus* sp. B264; curable; ~8 copies per chromosome	(73)
Selection markers	Kanamycin	50 μg/mL (selection) 250 μg/mL (plasmid function maintenance)	(85, 88, 97)
	Gentamicin	10 μg/mL	(82)
	Spectinomycin	100 μg/mL	(82)
	Thiostrepton	1 μg/mL	(97)
	Chloramphenicol	34 μg/mL	(98, 99)
	Hygromycin B	50 μg/mL	(73)
	SacB	Negative selection; sensitizes cell to sucrose	(100, 101)
Promoters	pTipA	Inducible with thiostrepton	(97, 102, 103)
	pAcet	5x inducible with acetamide	(98, 104)
	pBAD	59x inducible with arabinose	(104)
	pTet	67x inducible with anhydrotetracycline (aTc)	(104)
	pLPD06740	247x inducible with phenol	(104)
	pLPD06575	Inducible with phenol	(104)
	pLPD06699	39x inducible with phenol, protocatechuic acid, sodium benzoate, 4-hydroxybenzoate, vanillate, and guaiacol	(104)
	pLPD06568	80x inducible with phenol, sodium benzoate, and guaiacol	(104)
	pLPD03031	18x repressible with ammonium	(104)
	IGRI’ & IGRIV’	Inducible with 2,4-dinitrophenol (DNP)	(100)

Recombinases	Che9c60	GC-rich homologue of RecE	(73, 79, 105)
	Che9c61	GC-rich homologue of RecT	(73, 79, 105)
Neutral sites	ROCI-2	*R. opacus* chromosomal locus	(73)
	ROCI-3	*R. opacus* chromosomal locus	(73)
	ROP8I-1	*R. opacus* endogenous plasmid 8 locus	(73)

For promoters inducible with multiple chemicals, the highest published fold-change for a single compound is reported.

The ability to readily transform a bacterium with heterologous DNA is critical if it is to be a platform organism. *R. opacus* is transformable through multiple methods, including conjugation and electroporation. Conjugation requires a plasmid containing an origin of transfer (OriT) and a bacterial strain capable of conjugating with the strain of interest to horizontally transfer the plasmid (106). Both *E. coli* DH5α-pKOS111-47 and *E. coli* S17.1 have been used as conjugative helper strains with *R. opacus* (98, 100). Electroporation, wherein a pulse of electricity creates pores in the cellular membrane, can facilitate uptake of plasmid DNA in *R. opacus* at a reported efficiency of ~10^5 CFUs/μg DNA (97, 107).

Successful cellular engineering requires the use of well-characterized genetic parts for predictable gene expression, and in non-model organisms, parts are often borrowed from related organisms (108). One core component needed for reliable gene expression is the promoter, which drives gene transcription. A number of studies have utilized constitutive promoters from related gram-positive *Actinomycetales* (e.g. *Mycobacterium* spp. and *Streptomyces* spp.) or from genetically-distant bacteria, such as gram-negative *E. coli*, for heterologous gene expression (62, 63, 82, 88, 96). When performing metabolic engineering, however, a number of different promoters of varying strengths are required to balance the expression of multiple genes in an enzymatic pathway for optimal product titers (108, 109). An alternative to using borrowed promoters is the creation of a *de novo* constitutive promoter library, where many promoters of varying strengths are developed and characterized. Using a fluorescent reporter, a constitutive

promoter library spanning a 45-fold change in fluorescent output from weakest to strongest promoter was generated for *R. opacus* (73). Performing initial optimization of an enzymatic pathway combinatorically with a range of constitutive promoters, however, can be time-consuming and costly in non-model organisms.

An alternative to constitutive promoters is tunable promoters whose expression is induced or repressed relative to the concentration of a specific compound. Table 2 summarizes the inducible and repressible promoters that have been demonstrated in *R. opacus*. Of particular interest to the goal of using *R. opacus* for the conversion of lignin to lipids are the aromatic- and ammonium-responsive promoters. The aromatic promoters (pLPD06740, pLPD06575, pLPD06699, pLPD06568) are differentially induced in the presence of a variety of aromatic compounds, including some found in depolymerized lignin, and could be employed in metabolic engineering related to aromatic catabolism (104). pLPD03031 is a promoter that is repressed in the presence of ammonium, which can be used as a sole nitrogen source in *R. opacus*, and turns on when ammonium is depleted (104). As nitrogen starvation triggers lipid accumulation in *R. opacus*, this promoter could be used to modulate lipid pathways under lipid accumulating conditions (10, 54). Combining the aromatic- and ammonium-responsive promoters into genetic circuits could lead to dynamic regulation, which has been shown to increase final product titers through reductions in metabolic burden (29). Furthermore, pLPD03031 has been employed to create a cellular timer designed to activate at specific points in the cellular growth cycle, dependent on the initial ammonium concentration in the culture (104).

In addition to expressing a gene construct on a plasmid, heterologous expression can be achieved by integrating the DNA into the genome of the organism, where it can be stably maintained. To date, genome modification in *R. opacus* has been performed through both single-

and double-crossover homologous recombination (73, 98, 100, 105). Two methods utilizing single-crossover recombination, combined with the conjugative transfer of a donor plasmid via an *E. coli* helper strain, have been described for *R. opacus* (98, 100). One difficulty with genomic recombination in *R. opacus*, however, is that it often results in illegitimate integration, wherein the integration cassette is inserted at an incorrect location or the entire plasmid is integrated. This is a common issue in other actinobacteria, such as *Mycobacterium tuberculosis* and *Rhodococcus fascians*, and can be overcome through the heterologous expression of helper recombinases (79, 110). In *R. opacus*, a pair of bacteriophage recombinases, Che9c60 and Che9c61, have been demonstrated to facilitate double-crossover homologous recombination when donor template is provided via electroporation (73, 79, 105). Ideally, the integration of foreign DNA into the genome would have no adverse effects on cell health, but in practice care must be taken in choosing an integration site. Three neutral sites, or locations that have been demonstrated not to cause a decrease in growth rate when a gene cassette is integrated into them, have been identified in the chromosome and a native endogenous plasmid of *R. opacus* (73).

In addition to the tools that facilitate gene overexpression in *R. opacus*, it may also be desirable to eliminate certain genes. Genetic knockouts through homologous recombination have also been performed to disrupt gene expression. Both single- and double-crossover recombination have been used to knockout and confirm the functional roles of transcriptional regulators, catabolic enzymes, and transporters in *R. opacus* (9, 100). Furthermore, the *sacB* negative selection marker, which sensitizes the cell to sucrose, has been used for genome engineering in *R. opacus* (100, 101).

Gene knockouts can be informative when investigating gene function, but as permanent modifications, they may be lethal to the cell. As an alternative, a gene's expression can be selectively and temporarily reduced through CRISPR interference (CRISPRi). CRISPRi utilizes a

complex comprising a deactivated Cas9 (dCas9) and an engineered small guide RNA (sgRNA) to bind to DNA in a sequence-dependent manner and interfere with the transcriptional machinery, leading to targeted gene repression (111). The most commonly used CRISPRi system is derived from *Streptococcus pyogenes* ($dCas9_{Spy}$), but this system was found to be ineffective in *Mycobacterium tuberculosis*, an *Actinomycetales* species closely-related to *R. opacus* (112). A version of dCas9 sourced from *Streptococcus thermophilus* ($dCas9_{Sth1}$), which was found to be effective in *M. tuberculosis*, has been developed as a repression system for use in *R. opacus* (73). Experimentally, up to 58% repression of a chromosomally-integrated fluorescent protein was observed using this optimized $dCas9_{Sth1}$(73). Tunable gene repression using $dCas9_{Sth1}$ can be used in the future to remodel native metabolic pathways in *R. opacus*.

To quantify changes in gene expression between different growth conditions, stably-expressed reference (or "housekeeping") genes in *R. opacus* have been identified for use with reverse transcription quantitative PCR (RT-qPCR) (113). When ribosomal RNAs (rRNAs) are present in the samples, it was found that the combined use of genes for the ATP-binding subunit, ClpX, of the ATP-dependent Clp protease (PD630_RS25530) and 16S rRNA (PD630_RS01395) provided the best normalization results. If rRNAs are depleted, as is the case in samples prepared for RNA-Seq, the best pair of genes was found to be the same ATP-binding subunit ClpX and the rRNA small subunit methyltransferase G (PD630_RS37755). Using an appropriate set of reference genes is essential to generate meaningful expression-change data, and these pairs provide this baseline to an array of analyses.

A final technique that has been implemented to improve bioproduction in *R. opacus* is the development of a method for controlled cellular lysis to release intracellular compounds (114). A bottleneck in microbial manufacturing is separating the target product from the cells, and

implementing a controlled release strategy could reduce processing costs. A domesticated version of *R. opacus* created through serial culturing was found to be sensitive to a lytic tectivirus (Phage Toil) (114), which can be used to trigger cell lysis at a desired timepoint and can thus serve as a cheap and effective method for releasing products (e.g. TAGs) from *R. opacus* (114).

R. opacus as a production host

The production of TAGs and fatty acids in *R. opacus* has been demonstrated on an array of carbon feedstocks (Table 1-3). When fed kraft lignin (a toxic byproduct of the paper and pulping industry) in combination with laccase (a class of enzymes which oxidize phenolics), *R. opacus* was able to generate 0.145 g/L of lipid (59). A strain of *R. opacus* adaptively-evolved to tolerate higher levels of aromatic compounds, PVHG6, was able to generate 0.13 g/L lipids when provided with five lignin model compounds as sole carbon sources in equal quantities (9). Growth of *R. opacus* on pre-treated corn stover produced 1.3 g/L of lipid (measured as fatty acid methyl esters (FAME)) (60). A xylose-fermenting strain (MITXM-61) was developed by heterologous gene expression, and when it was grown in corn stover hydrolysates (containing 118 g/L initial total sugars), it converted xylose and glucose into 15.9 g/L TAGs (54% of dry cell weight (DCW)) (115). When *R. opacus* was cultured in glucose and glycerol, Suwaleerat et al. observed a maximum of 2.4 g/L lipids in 10.2 g/L biomass (116). While *R. opacus* grows poorly on glycerol alone (116, 117), this demonstrates that it can be used to enhance lipid production compared to just glucose as a sole carbon source. As glycerol is a byproduct of TAG transesterification, feeding it back to the production strain could reduce overall costs (118). Using adaptive evolution, Sinskey and colleagues generated an MITXM-61 derivative strain, MITGM-173, which was able to grow on up to 160 g/L glycerol (119). Optimized TAG production in this adapted strain occurred with a 1:2:2 mixture of glycerol:glucose:xylose, reaching 13.6 g/L TAGs (51.2% of DCW).

Table 1-3. Bioproduction by *R. opacus* wild-type and engineered strains on various feedstocks.

Strain	Substrate	Product	Production Value	Reference
R. opacus PD630	glucose:glycerol (7:3)	Carotenoids & lipids	0.99 mg/L & 2.4 g/L, respectively	(116)
R. opacus PD630	Pre-treated Corn Stover	FAME	1.3 g/L	(60)
R. opacus PD630	Glycerol	TAGs	1.4 g/L, 38.4% DCW	(117)
R. opacus PD630 (engineered)	Glucose	Fatty acids	46% DCW	(120)
R. opacus MITXM-61 (engineered)	Corn stover hydrolysates	TAGs	15.9 g/L, 54% DCW	(115)
R. opacus MITGM-173 (evolved)	glycerol: glucose:xylose (1:2:2)	TAGs	13.6 g/L, 51.2% DCW	(119)
R. opacus PD630	Crude Whey	Fatty Acids	45.1% DCW	(121)
R. opacus PD630	Switch Grass (pyrolysis oil)	Lipid	pH 7: 0.078 g/L, 21.9% DCW pH 4: 0.066 g/L, 25.8% DCW	(52)
R. opacus PD630	Kraft lignin (+ laccase)	Lipid	0.145 g/L	(59)
R. opacus PD630	Olive Mill Waste	Lipid	~1.9 g/L, 80% DCW	(122)
R. opacus PD630 PVHG6	phenol:vanillate:4-hydroxybenzoate: guaiacol:benzoate (1:1:1:1:1)	Lipid	0.13 g/L, 44% DCW	(9)
R. opacus PD630 (engineered)	Gluconate, whey	Wax esters	Gluconate: 46% total neutral lipids Whey: NR	(99)
R. opacus PD630	Poplar lignin hydrolysis slurry	Lipid	NR	(34)

FAME: fatty acid methyl ester; DCW: dry cell weight; NR: not reported.

R. opacus can also be used as a platform to produce high value compounds other than lipids. For example, *R. opacus* naturally produces carotenoids, which are prized for their pigmentation and antioxidant properties (116). In a 7:3 ratio of glucose and glycerol, *R. opacus* was able to produce 0.99 mg/L of carotenoids (116). Though *R. opacus* has, at present, a limited pool of demonstrated products (i.e., lipids and carotenoids), there is potential to expand the range, particularly as its genetic toolbox has recently been developed. With rational metabolic engineering, it may be possible to shunt more carbon flux into carotenoid production to improve yields and titers. Similarly, manipulation of enzymes in the β-ketoadipate pathway (Figure 1-1) could result in the accumulation of high-value intermediates, including *cis, cis*-muconic acid (bioplastic precursor) and succinic acid (food additive). Furthermore, the downstream product of aromatic degradation, acetyl-CoA, can be diverted to produce diverse compounds. Production of these high-value compounds from lignocellulose or lignocellulose-derived sources has been demonstrated in other bacterial hosts, which provides a guide for engineering *R. opacus* (Table 4).

Table 1-4. Compounds produced from lignin or lignin-derived sources in selected non-*R. opacus* bacterial hosts.

Strain	Substrate	Product	Production Value	Reference
R. jostii RHA1 (engineered)	Wheat straw	Vanillin	96 mg/L	(123)
P. putida KT2440 (engineered)	Depolymerized corn stover lignin	*Cis, cis*-muconic acid	3.7 g/L	(124)
Corynebacterium glutamicum (engineered)	Depolymerized softwood lignin	*Cis, cis*-muconic acid	1.8 g/L	(125)
Cupriavidus basilensis B-8	Kraft lignin	Polyhydroxyalkanoate (PHA)	319.4 mg/L	(126)
Actinobacillus succinogenes 130Z	Xylose-enriched corn stover hydrolysate	Succinic acid	39.6 g/L	(127)

Conclusion

Biofuels and bioproducts can be produced from lignocellulose to replace or supplement petroleum-based fuels, chemicals, and materials. To improve the economic competitiveness and reduce the environmental footprint of biorefineries, both the carbohydrate and lignin fractions should be utilized. However, due to its recalcitrance, lignin has been an untapped carbon source which is either discarded or burned for process heat. Additionally, aromatic compounds found in depolymerized lignin are toxic to most microbes, presenting a challenge to developing an economically-viable process. To overcome these challenges, we propose a hybrid conversion approach that combines thermochemical/catalytic and biological conversion processes as discussed in this review.

R. opacus is an ideal organism for such a hybrid conversion process due to its ability to tolerate and utilize a wide variety of aromatic compounds found in lignin breakdown products. Additionally, *R. opacus* is oleaginous and can produce high levels of lipids. While *R. opacus* engineering has been limited, recent identification of aromatic degradation pathways and substrate transporters has provided several targets that can be modified for strain optimization. In addition, the toolbox for genetic engineering is under active development, providing methods for gene modification and transcription control. Further engineering will be necessary to increase the tolerance, growth rate, and lipid production of *R. opacus* on depolymerized lignin substrates. Furthermore, *R. opacus* can be engineered to synthesize more valuable chemicals, such as branched-chain fatty acid esters, carotenoids, and *cis, cis*-muconic acid. With advances in both lignin depolymerization processes and rational engineering tool development for *R. opacus*, the coming years will witness rapid progress toward cost-effective conversion of lignocellulose into bio-products.

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Chapter 2: Application of transcriptomic data to achieve nitrogen-independent accumulation of fatty acids in *Rhodococcus opacus* PD630

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Abstract

Solving the global emissions crisis and reaching net-zero by 2050 will require multitudinous small changes to address the many facets of greenhouse gas production. One promising avenue in development is the replacement of conventional chemical and petrochemical production methods with biological solutions – production of value-added substances by microbial cell factories to either directly replace or substitute for the fuels, chemicals, and materials generated by standard means. Lignocellulosic biomass is being developed as a source of substrate for these bioproduction strains, but the recalcitrant lignin fraction represents a significant hurdle in efficient utilization. Whereas depolymerization of the cellulose and hemicellulose fractions yields sugar monomers which can be utilized by a broad spectrum of species, lignin is comprised of aromatic subunits which are toxic to many bacteria. *Rhodococcus opacus* PD630, an oleaginous soil bacterium, possesses the metabolic capacity to utilize lignin and its derivatives to amass high titers of triacylglycerols (TAGs), oleochemicals which can be used to generate biodiesel, detergents, lubricants, and other value-added products. Achieving the highest TAG accumulation, however, requires cells to be cultured in a state of nitrogen starvation, which is detrimental to growth, particularly on challenging aromatic substrates. Presented here is a transcriptome-based scheme to identify genetic regulators which promote TAG biosynthesis regardless of nitrogen condition. Several candidate genes were screened to find LPD06917, a regulator of phenylacetic acid degradation; when overexpressed, PD630 cells exceeded the baseline titer in both high nitrogen and phenol conditions. These

results provide a new framework for rational approaches to developing PD630 as a chassis for lignin valorization.

Introduction

For decades, climate scientists have been warning – with increasing urgency – that to preserve life on this planet, humanity must reduce carbon emissions; indeed, to limit the global average temperatures to within 1.5°C, it is estimate that carbon emissions must fall to a net zero by 2050, and more pressingly must be halved by 2030[1]. For all that individuals can and must do their part, the true change must happen on the scale of countries and entire industries. Compared to the energy sector (76% of greenhouse gas emissions in 2018), the 2.4% of emissions traceable to industrial chemical processing is a pittance, but it does account for more than a third of the chemical and petrochemical's total 6.4% of global emissions; transportation and assorted fuel combustion account for another 22.2% combined (14.2% and 8%, respectively)[2]. Utilization of biomass, especially lignocellulosic biomass, has the potential to disrupt emissions in all of these sectors – biofuels and commodity chemicals are commonly produced from the cellulose and hemicellulose fractions, but the lignin fraction is most frequently treated as a waste stream and consequently burned[3–5]. Lignin, however, is both energy-dense and has a high carbon-to-oxygen ratio (greater than 2:1), lending it immense promise as a feedstock for microbial bioproduction of biofuels and platform chemicals[5–8]. Achieving this bioproduction is not without its challenges – lignin is a complex, heterogeneous polymer, and the depolymerization process yields primarily aromatic compounds which are toxic to many microbes – but judicious choices in microbial platform and product could mean significant gains in the economic competitiveness of biorefineries, in addition to the emissions-related benefits[5,9–13].

Though model organisms can be engineered to degrade select aromatic compounds, perhaps a better starting point is a microbe that natively carries the capacity to tolerate and metabolize the products of lignin depolymerization. The eLignin database has indexed dozens of species across sixty-three genera which can natively degrade lignin or lignin-derived aromatics, with the two most-frequently represented genera being *Pseudomonas* and *Rhodococcus*[7]. Both genera include species with the breadth of catabolic potential particularly suited to the consumption of lignin and its breakdown products, but unlike most prokaryotes, *Rhodococcus* species have the capacity to produce triacylglycerols (TAGs) as a storage compound, rather than the more-common polyhydroxyalkanoates (PHAs)[8,14]. Some oleaginous species – including *R. opacus* PD630 (hereafter PD630) – may accumulate more than half of their dry cell weight (dcw) as TAGs under particular growth conditions[15–18]. TAGs and their derivatives comprise a substantial portion of industrial oleochemicals, ranging from soaps and detergents to biodiesel, so this is an area of bioproduction where finely-tuned microbial cell factories could make a significant impact [19–22]. Happily, PD630 is able to tolerate and utilize challenging feedstocks concurrently with amassing high lipid titers, including on several forms of lignin or lignin-derived feedstock[23,24].

Lipid production in oleaginous *Rhodococcus* species, particularly of TAGs, is largely linked to nitrogen stress: when nitrogen is depleted in the growth medium, cells store carbon in the form of TAGs, which can then be readily mobilized when the nutrient situation is reversed and carbon becomes scarce in the immediate environment[25]. This changeover makes sense on a stoichiometric level – in a paucity of nitrogen, synthesis of nucleotides and proteins must be metered, but with carbon available, the cells need not enter a state of suspension when they can shift production to compounds which can be assembled from the elements that are available (e.g.

carbohydrates and lipids). On a systemic level, however, linking lipid production to nitrogen starvation necessarily decouples it from growth, with most TAG accumulation corresponding to stationary phase[25]. This nitrogen dependence presents a challenge for bioproduction, especially from challenging carbon sources; lipid titers on aromatic substrates can be an order of magnitude lower than on more permissive ones (e.g. glucose, glycerol) because pairing the two stress conditions impairs growth, particularly in wild-type cells which have not been engineered or adapted for improved tolerance of either stressor[23,26].

Developing a strain for bioproduction is a delicate balancing act which can include locating targets for overexpression, adding novel functionality via heterologous genes, modulating gene regulation, and blocking competing pathways, all of which must be weighed against the potential for over-burdening the burgeoning cellular factory[22,27]. Broadly speaking, this process comes down to a design-build-test cycle which starts with identifying potentially-beneficial modifications. In *Rhodococcus*, most strategies to improve TAG production have focused on overexpressing native genes, including: increasing fatty acid synthesis with the *fasI* operon, boosting the final step in TAG biosynthesis with *atf2*, using thioesterases to increase fatty acid-CoA production, combining the long-chain fatty acid importer *ltp1* with growth on palmitic and oleic acids, and increasing the NAD(P)H pools via *tadD* or autologous malic enzymes[28–32]. While all of those have had a net positive effect on lipid accumulation in oleaginous rhodococci, none of them address the nitrogen-dependence of peak TAG production, nor the additional level of complication inherent to cultivating a production strain on a toxic feedstock like lignin or its byproducts. Maximizing the impact of a non-model microbial cell factory – like PD630 – depends upon optimizing yield in niches to which the

canonical strains (e.g. yeasts, *E. coli*) are not suited; achieving nitrogen-depletion levels of TAGs in nitrogen-replete, aromatic media would be a significant step in that direction.

Here, a transcriptome-based approach is used to identify overexpression and regulatory targets that can increase lipid titer in a nitrogen-independent manner, while also taking into consideration regulatory changes related to the presence of aromatic compounds in the media[33]. By re-examining transcriptome data for wild-type cells grown in phenol and under nitrogen starvation, a selection of genes which were differentially-expressed under both conditions were identified, and a subset were cloned into an overexpression vector. Following fermentation in permissive versus limiting carbon and nitrogen conditions, one overexpressed regulator was confirmed to promote fatty acid accumulation in a nitrogen-independent manner. Bioinformatic comparisons found a probable role in amino acid degradation, as well as several potential targets; the correct target was verified by knockout, which reverted to the wild-type lipid accumulation profile even when the overexpression vector was present. Taken together, these results both demonstrate that PD630 can achieve high lipid titer in nitrogen-replete conditions and improve understanding of the regulatory network response to nitrogen starvation. Additionally, this serves as a proof-of-concept study in identifying metabolic engineering targets via the transcriptome, which represents another tool for development of *Rhodococcus* as an efficient bacterial chassis for bioproduction from recalcitrant feedstocks.

Results

The nitrogen-dependent nature of fatty acid accumulation in *Rhodococcus* species is well-established, with both PD630 and related species switching from a growth mode to an oleaginous phenotype in response to low extracellular ammonium (NH_4^+)[25]. Additionally, while PD630 cells are more tolerant to aromatic carbon sources (e.g. phenol) than many bacteria,

they are not immune to the corresponding toxic effects, suffering a significant growth defect in, for example, phenol concentrations in excess of 0.75g/L[34]. When nitrogen depletion is combined with an aromatic feedstock, however, the combined stressors render the cells more sensitive to the toxic effects, resulting in a high titer of fatty acids relative to biomass which nonetheless totals to a low absolute quantity when the quantity of biomass is considered. As depicted in Figure 2-1, fatty acid quantities on a per-gram dry cell weight (dcw) basis are comparable at elevated carbon-to-nitrogen ratios ($C/N \geq 8$), indicating an independence from carbon source, but whereas nitrogen-starved cells fed 0.4g/L of glucose reach a combined dcw of ~30% that of cells in nitrogen-replete conditions, phenol-fed cells not only have a lower biomass titer, they are reduced to a mere 10% the density of their high-nitrogen counterparts. Decoupling fatty acid production from nitrogen depletion, therefore, may lead to greater overall production by removing one source of cellular stress and allowing fermentation cultures to proliferate more freely.

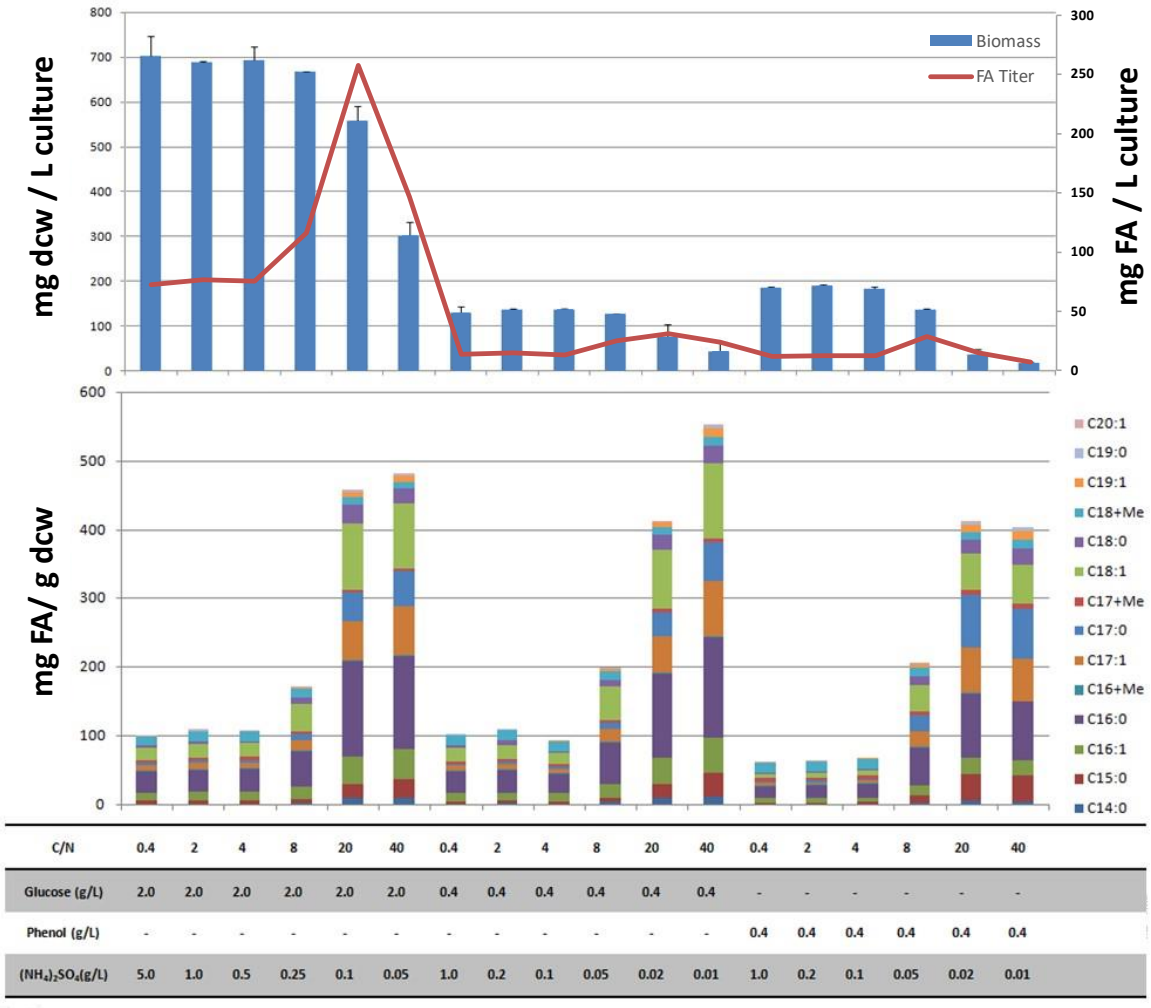

Figure 2-1. Nitrogen-dependent fatty acid (FA) content in Rhodococcus opacus PD630 is independent of carbon source, in counterpoint to toxic effects on biomass. As the carbon-to-nitrogen ratio (C/N above) increases to 8 and above, FA accumulation in WT PD630 cells increases dramatically on a per-milligram dry cell weight (dcw) basis, at a cost to overall biomass accumulation and consequently FA titer. While cells provided with phenol can reach comparable FA levels (as a fraction of dcw) to cells fed with glucose, the impact on biomass is more pronounced due to the compounded stresses of nitrogen depletion and feedstock toxicity, displaying an approximately 90% reduction in biomass from nitrogen-replete to nitrogen-depleted conditions, versus a 70% reduction in cells provided the same initial concentration of glucose.

Two previously-published transcriptomic datasets were revisited to identify transcription factors which respond to both nitrogen and phenolic stress, which could then be overexpressed and screened for nitrogen-independent fatty acid production[26,35]. Of the 399 regulator genes reviewed, 141 responded with at least a two-fold change in transcription (either up- or down-

regulation in comparison to cells cultivated in rich media) to phenol, and 216 responded to low nitrogen; this pool was narrowed to 33 candidates which responded to both stimuli, at least one of which was a strong (>2-fold) response Figure 2-2A. These transcription factors were cloned into the high-copy pAL5000(S) backbone under a strong constitutive promoter; 30 plasmid constructs were confirmed by sequencing, and 27 were successfully overexpressed in PD630 (see Supplementary Table 1 for a full list)[36]. A final subset was selected for fatty acid fermentations to represent the four possible combinations of up- and down-regulation in response to the two stimuli of interest. For the fermentations, the low nitrogen concentration (0.05g/L $(NH_4)_2SO_4$) was chosen to optimize fatty acid titer in phenol cultures, and the high-nitrogen condition (1.0g/L $(NH_4)_2SO_4$) is sufficient for nitrogen-replete growth and fatty acid accumulation in both carbon conditions; optimal FA accumulation in glucose was not required. In comparison to the wild-type (WT) expressing an empty vector control, the pWG013 strain (overexpressing the LPD06917 locus) produced a higher titer of fatty acids in three of four fermentation conditions – 2.0g/L glucose with 1.0g/L $(NH_4)_2SO_4$, and 0.4g/L phenol with both 1.0 and 0.05g/L $(NH_4)_2SO_4$ – and more importantly did not display nitrogen dependence in its fatty acid accumulation; additionally, in the dual-stress condition of phenol with nitrogen depletion, the pWG013 strain's fatty acid titer was substantially higher than all other strains tested Figure 2-2B. As the primary motivation behind achieving nitrogen-independent TAG accumulation is to conduct TAG-producing fermentation of PD630 in aromatic (e.g. lignin-based) media without the growth defect observed under nitrogen stress, observing an increase in fatty acid titer under the phenol/high nitrogen condition is sufficient justification to move forward with examining the regulatory effects of overexpressed LPD06917 in the pWG013 plasmid.

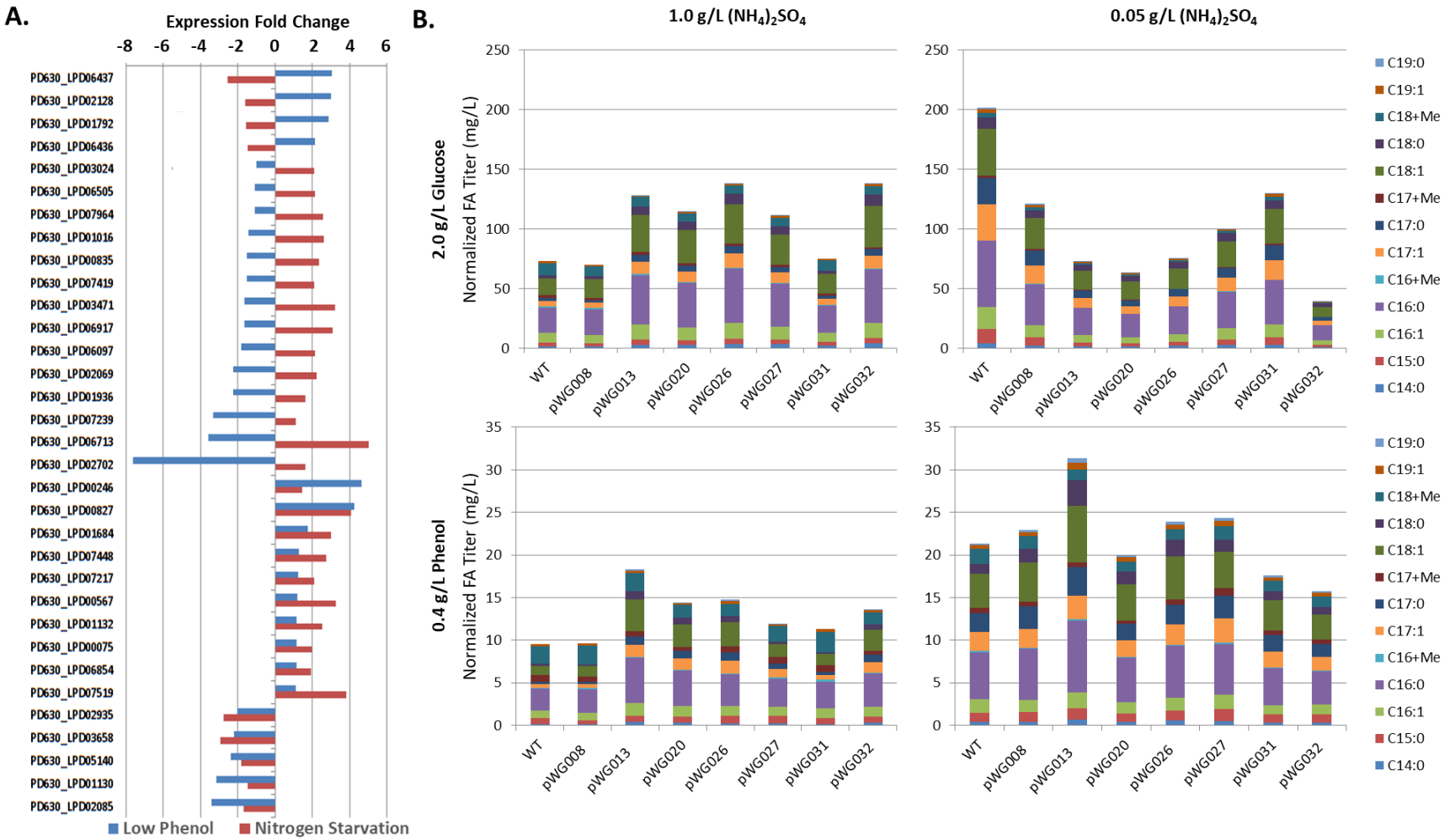

Figure 2-2. Transcription regulators with strong differential expression under phenol and nitrogen starvation conditions were overexpressed in Rhodococcus opacus PD630 to identify factors influencing nitrogen-independent fatty acid (FA) accumulation. **(A)** Previously-published transcriptome datasets were mined to identify regulator genes which are differentially-expressed in both phenol and nitrogen starvation (greater than one-fold change in expression) versus in nutrient broth (NB); this pool of genes was narrowed to 33 by selecting only those which exhibited a greater than two-fold change in expression to at least one of the conditions. Of these 33, 27 were successfully overexpressed in PD630; for full details, see Supplementary Table 1. **(B)** A subset of overexpression strains, representing the four possible combinations of differential gene expression, were selected for FA fermentation; strains were grown in glucose or phenol, and nitrogen-rich or –starved media for 60 hours, then the lipids were extracted and analyzed by GC-FID. The pWG013 strain, overexpressing LPD06917, produced more FA than the WT in three of four growth conditions, and exhibited the highest FA titers of all tested strains in low-nitrogen media with phenol. All fatty acid titers have been normalized to an internal 40mg/L C_{12} standard and averaged across technical replicates.

To elucidate how LPD06917 fits into the transcriptional network, and thus characterize the effect of the pWG013 overexpression plasmid, bioinformatic comparison of the gene was used to identify both a probable function and the likely target operon. LPD06917 is annotated as PaaX, a negative regulatory protein acting on the phenylacetic acid degradation operon, or Paa gene cluster. This genes of this cluster, which spans loci from LPD07014 to LPD07028, are modestly downregulated in low concentrations of phenol, and highly downregulated under nitrogen starvation; LPD06917, as one might expect of the corresponding negative transcription factor, is upregulated in response to nitrogen starvation (Supplementary Figure 1). To identify the specific target of this transcriptional regulator within the cluster, three knockout strains were generated: ΔLPD07016, ΔLPD07015, and ΔLPD07014. These three loci represent the start of the pheylacetic acid degradation pathway, and were chosen because regulation of an entire fifteen-gene cluster is more parsimoniously accomplished by downregulating its initial step(s) than acting on every step, or even a selection of steps downstream in the pathway. The LPD07016 locus encodes a ligase responsible for catalyzing the conversion of phenylacetate to phenyl-acetyl-CoA, and thus could be considered the first step of the phenylacetate degradation operon; LPD07015, which is immediately downstream and shares regulatory elements with LPD07016, encodes a feaR-type regulator which may act on other genes of the cluster. Both genes are plausible targets of PaaX. Additionally, due to the shared promoter region, knocking out LPD07016 also disrupts LPD07015 expression, so a separate ΔLPD07015 strain enables precision in pinpointing which gene is regulated by PaaX. LPD07014 – which encodes an amidase that hydrolyzes 2-phenyl-acetamide into phenylacetate – has similar transcriptional behavior to the other Paa genes, but acts as a precursor step to the rest of the cluster. Repressing LPD07014, however, would not be as effective in shunting flux away from the Paa cluster due to

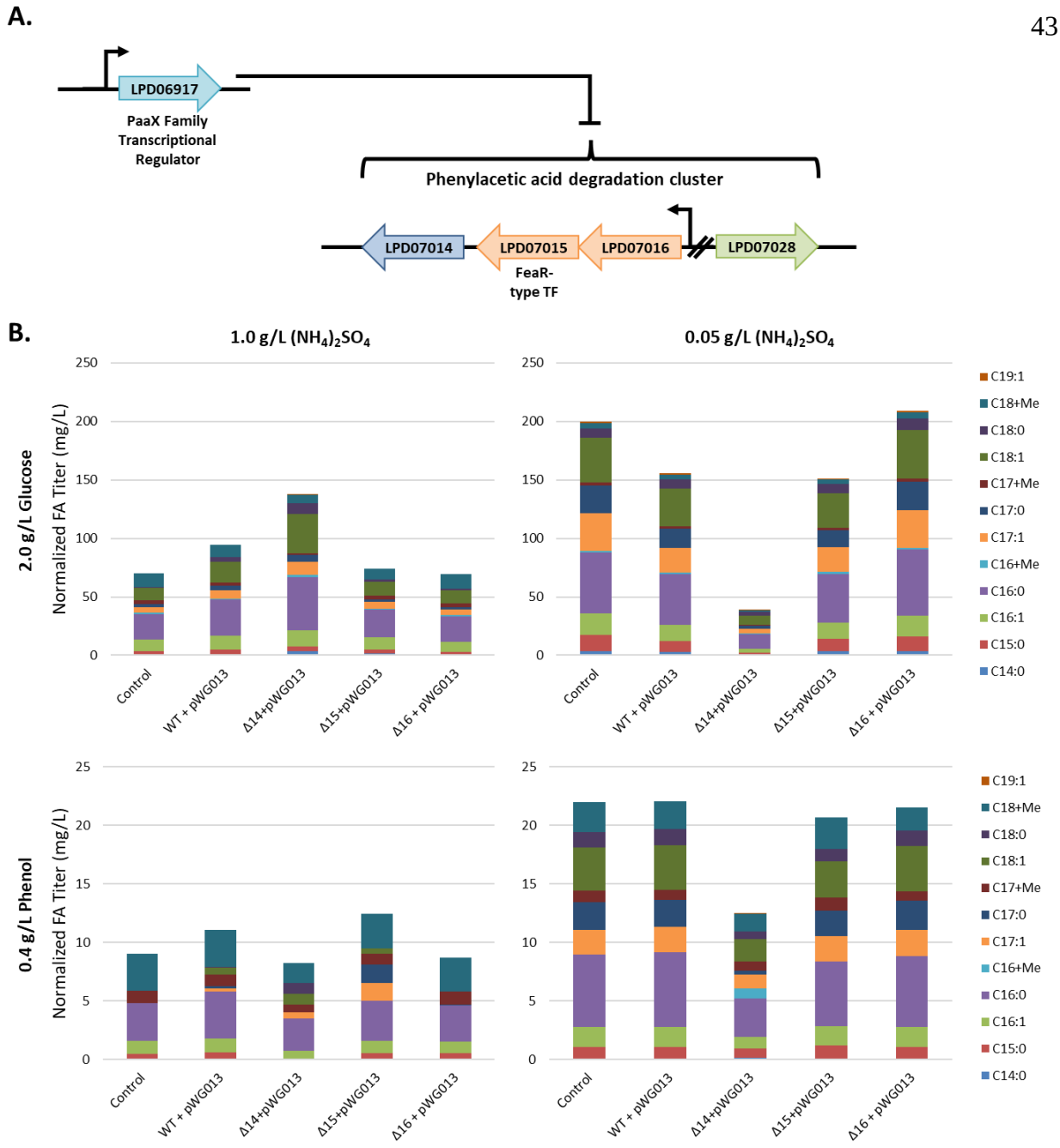

Figure 2-3. Transcriptional regulation of the phenylacetic acid degradation cluster by PaaX modulates fatty acid accumulation in PD630. (A) Bioinformatic analysis identified LPD06917 as encoding a PaaX family negative regulatory protein; the phenylacetic acid degradation (Paa gene cluster; see Supplementary Figure 1 for details) loci LPD07015 and LPD07016, which are controlled by a shared promoter, as well as LPD07014 immediately downstream, are proposed to be the most likely targets. Promoters are represented as small arrows, and genes are shown with LPD gene numbers from the NCBI database (Refseq, CP003949.1). **(B)** Fatty acid (FA) titers (mg/L) under standard fermentation conditions for wild-type (WT), ΔLPD07014 (Δ14), ΔLPD07015 (Δ15), and ΔLPD07016 (Δ16) strains carrying the LPD06917 overexpression plasmid (pWG013), in comparison to a positive (WT + pWG013) and negative (Control) controls. All fatty acid titers have been normalized to an internal 40mg/L C_{12} standard, averaged across technical replicates, and transformed to standardize the summed control titers between fermentation experiments.

from phenylacetaldehyde – so LPD07015 and LPD07016 were considered the most likely targets of PaaX. Figure 2-3A

The three knockout strains were transformed with pWG013, then fermented under standard conditions in comparison with both positive (WT + pWG013) and negative (WT expressing an empty vector) control strains Figure 2-3B. When LPD07016 was knocked out, the presence of pWG013 had no effect on fatty acid titer, with no change observed versus the negative control. Knocking out LPD07015, however, maintained the LPD06917 overexpression phenotype, with fatty acid titers approaching those of WT + pWG013 in all conditions. Taken together, these trends suggest that PaaX regulates LPD07016, but not LPD07015. Unexpectedly, the data for ΔLPD07014 + pWG013 indicates that there is some interplay between PaaX and LPD07014. In the absence of both nitrogen and aromatic stress, ΔLPD07014 + pWG013 generated more fatty acids than either of the control strains, and when phenol was provided, its titers were a match to the empty-vector control. Under nitrogen depletion, however, this strain exhibited a dramatic reduction in fatty acid accumulation, suggesting the balance between LPD06917 and LPD07014 expression is crucial to low-nitrogen fatty acid biosynthesis. Fatty acid synthesis was not perturbed in the baseline ΔLPD07014 strain, indicating that this balance relies more heavily on LPD07014 than it does on LPD06917 (Supplementary Figure 2).

Discussion

Rhodococcus opacus PD630, while not a conventional bioproduction strain, has several traits that position it well for development as a specialist microbial cell factory. Its native catabolic capabilities are diverse, allowing it to grow on a collection of challenging substrates, in particular aromatic compounds. As lignin is almost exclusively comprised of aromatic units, PD630 is positioned ideally as a biological platform for lignin valorization, especially with

ongoing efforts to improve catabolic repertoire, tolerance of toxic substrates, and ability to handle unprocessed or minimally-processed lignin[28,34,37–40]. Lignin or lignin-derived compounds have been used as a feedstock to produce biological storage compounds (TAGs, PHA) and metabolic intermediates (vanillin, 4-hydroxybenzoate, *cis, cis*-muconic acid) by various bacteria, but most work in the oleaginous PD630 focuses on improving the yield of various lipids[5,23]. Much of this research has started from a particular carbon source and tweaked culture conditions or genomes to optimize lipogenesis, but the only change found to reduce or mitigate the nitrogen-dependence of fatty acid in *Rhodococcus* was incidental to an investigation into the role of the actinomycetes-exclusive regulator NlpR[41]. The elevated carbon-to-nitrogen ratio which supports optimal lipid production in cells without overexpressed *nlpR*, however, is detrimental to growth of cells in culture, particularly with aromatic substrates. This work seeks to uncover additional regulatory gene targets which can be modulated to decouple nitrogen availability and lipid production in support of improving TAG production from lipid-derived feedstocks.

The results described herein demonstrate proof-of-concept for transcriptome-driven strain design in PD630, as well as identify the first genetic modification which improves TAG production in nitrogen-replete conditions. Additionally, insight is gained into how PD630 rebalances the metabolic network to support high-titer TAG production. This work supports the development of PD630 as a chassis for lignin-derived lipid production, and lays groundwork for how transcriptomic data can be used in the future to support strain optimization, linking into the ‘design’ facet of the design-build-test cycle of rational strain engineering.

To date, investigations of TAG biosynthesis in oleaginous *Rhodococcus* (both PD630 and the closely-related strain *R. jostii* RHA1) cultures have been fragmentary and focused primarily

on systemic changes, like changes in carbon flux and regulation of major pathways, or on evaluating the role of specific genes putatively identified as being directly related to known lipid biosynthesis pathways[42,43]. Regulation occurs at a transcriptional level, with the nitrogen-sensing protein GlnR upregulating NlpR to in turn regulate FASI, FASII, and aspects of lipid metabolism, and at the substrate level, with competing pathways like glycogen synthesis being inhibited by NADPH and Entner-Doudoroff (ED) pathway intermediates[41,44]. NADPH accumulation, however, is at least in part the result of upregulation of a non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase called TadD, which is specifically induced during TAG accumulation; similarly, the ED pathway and pentose phosphate (PP) pathway (another source of NADPH) are highly upregulated under conditions consistent with TAG accumulation[45,46]. While there are many factors which have not been elucidated yet, including more specific regulation of lipogenesis, it is clear that transcriptional regulation plays a major role in TAG synthesis, and thus is a suitable target for investigating factors that influence the nitrogen-dependent aspect thereof.

While the concept of exploiting transcriptomic datasets to identify differentially-expressed genes which may influence the development of a chosen phenotype – in this case, nitrogen-independent TAG accumulation – is sound, the paucity of data for PD630 likely influenced the accuracy of the predictions made here. Of seven overexpressed regulators screened in PD630, only one precipitated the desired phenomenon. Assumptions made about the comparability of the two datasets may be to blame – the Chen et al (2014) and Yoneda et al (2016) data both examined PD630 grown in minimal salts media with a 20:1 carbon-to-nitrogen ratio, but the comparability of TAG accumulation in differing carbon sources at the same ratio may not translate to comparable transcription profiles[26,35]. Additionally, the two conditions

examined from the Yoneda et al (2016) data – nitrogen starvation and low phenol – were both conducted at a nitrogen concentration of 0.05 g/L $(NH_4)_2SO_4$, with either 1.0 g/L glucose or 0.75 g/L phenol; while 15:1 carbon-to-nitrogen is not at the level of nitrogen starvation where the highest TAG accumulation is observed, it crosses the threshold where cellular priorities are switching away from biomass and toward lipogenesis. In comparing them to the rich-medium data from Chen et al (2014), the influence of phenol was thought to outweigh that of nitrogen depletion, but with the data available it is not possible to truly decouple the two factors. It is possible that, with datasets generated from growth conditions that are more directly comparable and examine the variables of interest independently, a higher rate of accuracy is possible in predicting regulatory factors relevant to nitrogen-independent TAG production.

Though the success rate of the transcriptome-guided search method is, as described here, underwhelming, LPD06917 was positively identified as a factor relevant to the nitrogen-dependence of TAG accumulation. The full details of this effect nevertheless remain cryptic. Bioinformatic comparison identifies LPD06917 as encoding PaaX, a negative regulator of the phenylacetic acid degradation operon or Paa cluster. This operon is a component of phenylalanine metabolism which ultimately produces acetyl-CoA – a critical precursor to lipogenesis – so it follows that this pathway, like the TCA cycle, might be prioritized when TAG production is favored[47]. This hypothesis runs counter to the annotated role of LPD06917, as well as the correlation between its upregulation in low nitrogen and the Paa cluster's downregulation in the same. To that end, preservation of the phenylalanine pool may be at play – the oleaginous yeast *Yarrowia lipolytica* downregulates amino acid biosynthesis during nitrogen limitation, and there is evidence that *R. jostii* RHA1 upregulates genes related to amino acid metabolism under nitrogen starvation[46,48,49]. Specifically, a shikimate 5-dehydrogenase

is upregulated to produce NADPH, which would decrease phenylalanine, tyrosine, and tryptophan synthesis; if this also occurs in PD630, downregulation of the Paa cluster may prevent the pool from being entirely depleted[46]. Overexpressing LPD07016, the apparent target of LPD06917, may clarify the situation – a comparable increase in fatty acid titer may indicate that LPD06917 is functioning as an activator, but the development of a growth defect, particularly in low nitrogen conditions, may suggest that the Paa cluster is instead being downregulated to maintain phenylalanine availability. As such, while this study has provided some insight into the role of the Paa cluster vis-à-vis TAG accumulation in low-nitrogen conditions, there is more still to uncover with further experimentation.

Described here is an initial investigation into the use of transcriptome data to locate likely targets for nitrogen-related control of TAG synthesis in PD630, and the identification of one such regulatory element. While these results provide early validation of the method, there is room for improvement, and revisiting the other loci successfully cloned into PD630 may yield additional hits. Nonetheless, by identifying the LPD06917 locus as a factor in low-nitrogen TAG accumulation, the number of genes which can be overexpressed to improve TAG titers in high-nitrogen conditions has effectively doubled. By combining these findings with additional methods of boosting lipogenesis and aromatic tolerance, it may be possible to develop a TAG production chassis suitable for use with recalcitrant, lignin-derived aromatic feedstocks.

Materials and Methods

Chemicals & Strains

Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO). The ancestral, or wild-type (WT), strain for all transformant cells lines was *Rhodococcus opacus* PD630 (DSMZ 44193); this strain was used as a basis of comparison for all transgenic

strains. Culturing conditions for all experiments, unless otherwise noted, were incubation at 30°C with 250rpm shaking, with the previously-described minimal salts medium B constituting the growth medium[50]. Media was sterilized using a 0.22µm filter, with carbon sources added as filter-sterilized stock solutions; nitrogen was added either pre-sterilization or as a separate filter-sterilized stock solution. Media pH was adjusted to 7.2 using 6N HCl or 2M NaOH solutions. Optical density at 600nm (OD_{600}) was measured using a Tecan Infinite 200Pro plate reader, either directly using VWR semi-micro polystyrene cuvettes or indirectly based on the absorbance at 600nm (A_{600}) measured in black 96-well plates (Greiner Bio-One flat bottom, chimney well, µclear); an A_{600} value can be converted into an OD_{600} value (for *R. opacus* cultures) via the experimentally-determined relationship $OD_{600} = 1.975 \times (A_{600} - 0.04)$.

All strains were maintained on tryptic soy broth (TSB) plates supplemented with 1.5% agar. Kanamycin (20µg/mL), gentamicin (10µg/mL), chloramphenicol (34µg/mL), or hygromycin B (200µg/mL) was added as appropriate to *E. coli* cultures. Kanamycin (50µg/mL), gentamicin (10µg/mL), chloramphenicol (15µg/mL), and/or hygromycin B (50µg/mL) were added as appropriate to *R. opacus* cultures.

Plasmid Construction and DNA Manipulation

All plasmids constructed for this study were confirmed by DNA sequencing (Genewiz; South Plainfield, NJ); all primers were purchased from Integrated DNA Technologies (IDT; Coralville, IA). All overexpression plasmids were assembled using GoldenGate Assembly, and knockout plasmids were assembled using Gibson Assembly; all plasmids were replicated in *E. coli* DH10B, then isolated using a PureLink™ HiPure Plasmid Miniprep Kit (Invitrogen by ThermoFisher; Waltham, MA)[51,52]. DNA fragments amplified were amplified using Phusion High-Fidelity DNA Polymerase (NEB; Ipswich, MA) and purified using a ZymoClean Gel DNA

Recovery Kit (Irvine, CA); genomic DNA was extracted from *R. opacus* using a Promega Wizard™ Genomic DNA Purification Kit (Madison, WI).

Transformation of *R. opacus*

Preparation of competent cells was conducted as previously described[50]. In brief, an overnight culture in TSB medium was used to inoculate 100mL of fresh TSB containing 8.5g/L glycine and 10g/L sucrose (initial optical density, OD_{600}, diluted to 0.075); cells were cultivated in standard conditions to an OD_{600} value of 0.4-0.5, corresponding to exponential phase growth. Cells were rapidly chilled and centrifuged at 3.5k relative centrifugal force (rcf), washing twice with chilled, sterile, deionized water; a final resuspension to an OD_{600} ~ 10-15 was conducted in chilled 10% (v/v) glycerol, and cells were aliquoted at 100µL and frozen at -80°C for transformation.

For transformation with a replicating plasmid, approximately 500ng plasmid DNA was added to prepared electrocompetent cells; cells were shocked at 2500mV across a 0.2cm-gap cuvette (time constant ~5-6ms) and washed with 1mL rich media (either TSB or SOC/super-optimal broth with catabolites) to recover. For outgrowth, cells were transferred to 50mL glass culture tubes and incubated under standard growth conditions for 4 hours before being spread on TSB plates infused with the appropriate antibiotics. Plated cells were grown at 30°C for 2-3 days, until colonies emerged, then propagated on fresh plates.

Generation of *R. opacus* knockout mutants was accomplished using a previously-developed method for homologous recombination, with modifications[36]. Briefly, electrocompetent cells were prepared as above using a strain expressing a helper plasmid containing the Che9c viral recombinases. Competent cell aliquots were transformed with 1-2µg of a suicide vector containing the knockout construct (an antibiotic resistance cassette flanked by

~500bp segments homologous to the target gene); the outgrowth period for these transformations was at least 6 hours and up to 12 hours. Transformed cells were plated on TSB with the corresponding antibiotics and incubated at 30°C for 4-5 days; colonies were propagated on fresh TSB plates and verified by colony PCR (Promega GoTaq® G2 DNA Polymerase).

Fermentation for Lipid Analysis

Frozen stocks of strains (generated from isolated colonies and stored at -80°C) were streaked onto fresh TSB plates, with antibiotics as appropriate, then grown for 2-4 days; a loopful of cells were used to inoculate seed cultures in minimal media B with 1g/L each $(NH_4)_2SO_4$ and glucose as nitrogen and carbon sources, respectively[50]. These cultures were centrifuged at 3.5k rcf and the pellets resuspended in low-nitrogen minimal media; OD_{600} of these cell suspensions was adjusted to approximately 2, then used to inoculate the 50mL fermentation cultures (250mL non-baffled Erlenmeyer flasks). The carbon conditions were either 2g/L glucose or 0.4g/L phenol, and the nitrogen conditions were either 0.05g/L or 1g/L $(NH_4)_2SO_4$ (hereafter 'low' and 'high' nitrogen), for a total of four combinatorial conditions; each strain was grown in one flask per carbon/nitrogen condition. Cultures were grown in standard conditions for 72 hours unless otherwise described; final OD_{600} was measured and used to calculate the volume necessary to collect 5 OD units of cells (or 10 OD units, in the case of the glucose/low nitrogen condition) using the relation $Vol = 5/OD_{600}$. Triplicate samples of each strain and culture condition were collected and centrifuged at 3.5k rcf for 10 minutes, then the culture supernatant was discarded and the cell pellets were stored at -20°C prior to lipid extraction.

Lipid Extraction & Analysis

An acid-chloroform lipid extraction was performed as described in Amara et al (2016), with modifications. In brief, the pelleted 5 (or 10) OD units of cells were resuspended in 100µL sterile, deionized H_2O and transferred to a 15mL glass centrifuge tube. A 1mL aliquot of 10% (v/v) H_2SO_4 in methanol was added to the cells, as well as 1mL of chloroform and a C_{12} standard (40mg/mL lauric acid dissolved in methanol) to a final concentration of 40mg/L. Cell solutions were incubated at 100°C for one hour, then chilled rapidly on ice prior to adding 1mL of deionized H_2O and mixing thoroughly by vortex. Finally, the cell extracts were centrifuged at 1k rpm for 5 minutes at room temperature, and the resulting organic layer was extracted into GC vials.[49] Samples were stored at 4°C prior to analysis.

Lipid extracts were analyzed via GC-FID using an Agilent 6890N Network GC system with Enhanced Agilent MSD ChemStation software and equipped with a 7683B series injector, G2614A autosampler, and DB-5ms column. A 1µL sample of each lipid extract was splitlessly injected into the 250°C inlet, with N_2 carrier gas flowing at 1.4mL/min. Each sample run began with an initial oven temperature of 80°C, then ramped at 20°C/min to 300°C, where it held for a final three minutes. Between samples, the injector was washed with ethyl acetate, with ethyl acetate also serving as the blank samples at the start and end of each sample run. Peak integration was carried out using the ChemStation software and exported to Microsoft Excel for data processing.

In addition to the C_{12} internal standard, lipid samples were compared to standard curves for thirteen other fatty acids, detailed in Table 2-1. All standard curves plotted the area of the peaks of 25mg/L, 50mg/L, and 100mg/L standard compounds. The fitted slopes of these curves were used to calculate the concentration of each component within the extracted lipid samples,

matched by retention time and normalized to the 40mg/L lauric acid internal standard. If necessary for comparison, summed average titers were transformed to standardize control samples between different fermentation experiments.

Table 2-1. Fatty acid standards for analysis of lipid samples extracted from *Rhodococcus opacus* PD60 cells.

Short Notation	Long Notation	Linear Formula	Retention Time (min)
C12:0	lauric/dodecanoic acid	$CH_3(CH_2)_{10}COOH$	6.23
C14:0	myristic/tetradecanoic acid	$CH_3(CH_2)_{12}COOH$	7.388
C15:0	pentadecanoic acid	$CH_3(CH_2)_{13}COOH$	7.929
C16:1	palmitoleic/hexadecenoic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	8.351
C16:0	palmitic/hexadecanoic acid	$CH_3(CH_2)_{14}COOH$	8.443
C16+Me	methyl palmitate/ hexadecanoate	$CH_3(CH_2)_{14}CO_2CH_3$	8.624
C17:1	heptadecenoic acid	$CH_3(CH_2)_5CH=CH(CH_2)_8COOH$	8.804
C17:0	margaric/heptadecanoic acid	$CH_3(CH_2)_{15}COOH$	8.938
C17+Me	methyl margarate/ heptadecanoate	$CH_3(CH_2)_{15}CO_2CH_3$	9.12
C18:1	oleic/octadecenoic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	9.302
C18:0	stearic/octadecanoic acid	$CH_3(CH_2)_{16}COOH$	9.408
C18+Me	methyl stearate/octadecanoate	$CH_3(CH_2)_{16}CO_2CH_3$	9.57
C19:1	nonadecenoic acid	$CH_3(CH_2)_7CH=CH(CH_2)_8COOH$	9.74
C19:0	nonadecanoic acid	$CH_3(CH_2)_{17}COOH$	9.862

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Chapter 3: Conclusion

It is an unassailable fact that fossil fuels have made an indelible mark on humanity, allowing progress in technology, agriculture, infrastructure, science, and public health to be made in leaps and bounds. It is an equally valid fact that the planet is on fire – an almost imperceptibly small fire, but a fire nonetheless – and that the phenomenon can be tracked to the cumulative greenhouse gases emitted by a highly-industrialized global society. To hold the planet to a mere 1.5°C above pre-industrial average global temperatures, the roughly 50 gigatons of net CO_2 equivalents admitted into the atmosphere annually must be halved by 2030, and entirely eliminated by 2050. The are no simple fixes – to solve this, humanity must take apart the problem bit by bit and solve it in small parts until the cumulative effect reaches the scale of the whole. One of these small solutions is the valorization of lignin biomass into value-added compounds.

Lignin, the most recalcitrant of the three lignocellulosic biomass components, is a complex heteropolymer comprised of highly-variable ratios of aromatic alcohols. Unlike cellulose and hemicellulose, which can readily be depolymerized into sugar monomers, the process of breaking lignin down into constituent monomers is complex and inconsistent batch-to-batch. Additionally, the costs associated with separating out useful and less-useful compounds from breakdown products are high enough that, in most cases, lignin is either discarded as waste or burned to generate energy. There is an alternative – certain microbes, including *Rhodococcus opcaus* PD630, have the breadth of catabolic processes necessary to metabolize lignin and its aromatic breakdown products. Aromatic-consuming microbes break down lignin-derived feedstocks into central metabolites, which can then be fed into different production pathways, including lipid metabolism. PD630, in particular, is an oleaginous bacterium capable of

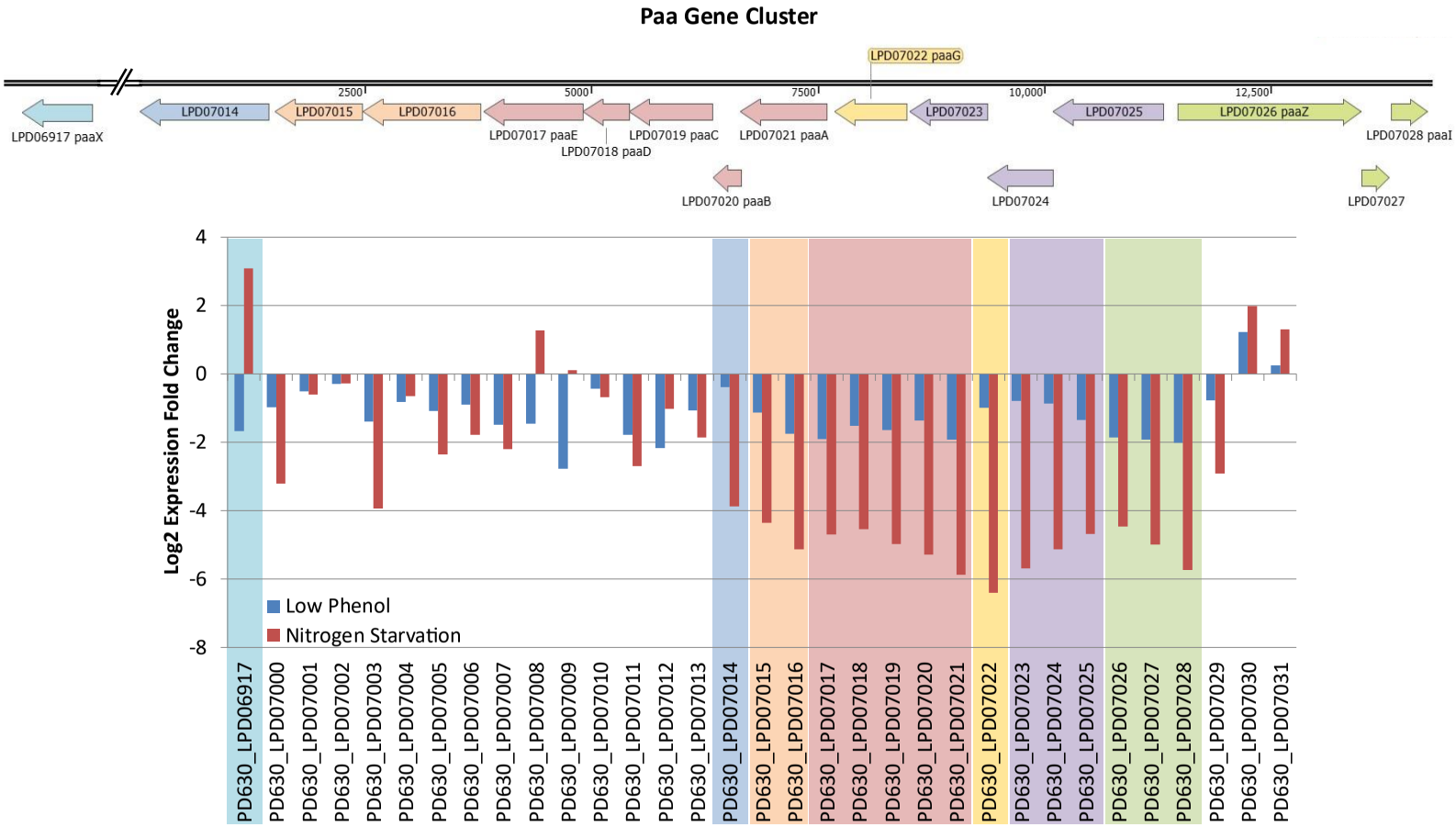
accumulating over 75% of its dry cell weight (dcw) as triacylglycerides (TAGs) under optimal conditions. TAGs are oleochemicals readily converted into an array of products, including biodiesel, detergents, food additives, lubricants. Additionally, because PD630 is natively highly tolerant to aromatic compounds that are toxic and inhibitory to more conventional production strains, it is able to be cultured on higher substrate loads and achieve higher titers of a given product.

The research described in this thesis focuses on the development of PD630 as a microbial chassis for biocatalysis of renewable biomass into value added compounds, concentrating in particular on lipid production. The primary condition affecting lipogenesis and TAG accumulation in oleaginous rhodococci is nitrogen limitation, with production of this storage compound depending strongly on the ratio of carbon-to-nitrogen in the extracellular environment. TAG synthesis competes with cell growth, however, and comes at a cost of decreased tolerance to aromatic stress. In support of optimizing conditions for microbial cell factories converting lignin-derived biomass into high TAG titers, this work explores regulatory factors which respond to low-nitrogen and aromatic stimuli in search of elements which could contribute to decoupling nitrogen starvation from TAG production. The findings provide insight into the metabolic rearrangement that occurs during the changeover from growth to a TAG accumulation phenotype, and the methods used to identify candidate regulators could, with appropriate transcriptomic data, be applied to mine the genome for loci relevant to an array of environment-dependent phenotypes. In sum, this project supports the development of PD630 as a bioproduction chassis for lignin valorization and lays the groundwork for additional improvements.

Appendix: Supplementary Information for Application of transcriptomic data to achieve nitrogen-independent accumulation of fatty acids in *Rhodococcus opacus* PD630

Supplementary Table 1. Overexpression strains screened for nitrogen-independent fatty acid production in *Rhodococcus opacus* PD630.

Overexpression Strain	Locus ID	Gene Name
PD630 + pWG001	LPD06713	yagI
PD630 + pWG002	LPD02702	liaR
PD630 + pWG005	LPD01792	degU
PD630 + pWG006	LPD06436	dosT
PD630 + pWG008	LPD02128	yobV
PD630 + pWG009	LPD07239	ybhD
PD630 + pWG012	LPD06097	BetI
PD630 + pWG013	LPD06917	paaX
PD630 + pWG014	LPD03471	yidP
PD630 + pWG015	LPD07419	ycbG
PD630 + pWG016	LPD00835	ttgR
PD630 + pWG018	LPD07964	yiaJ
PD630 + pWG019	LPD06505	kipR
PD630 + pWG020	LPD3024	Rv0472c-MT0489
PD630 + pWG024	LPD00827	ttuE_C
PD630 + pWG025	LPD01684	yxaF
PD630 + pWG026	LPD00567	tcmR
PD630 + pWG027	LPD07519	slyA
PD630 + pWG028	LPD02935	Leu
PD630 + pWG029	LPD03658	HI_1364
PD630 + pWG030	LPD01130	pro_trans
PD630 + pWG031	LPD02085	NarL
PD630 + pWG032	LPD07217	ttgR
PD630 + pWG033	LPD05140	Mb0601
PD630 + pWG034	LPD01132	yagI
PD630 + pWG035	LPD00075	ribonuclease
PD630 + pWG036	LPD06854	famr



Supplementary Figure 1. Phenylacetic acid degradation (paa) gene cluster overview and transcriptional regulation in low phenol and nitrogen starvation conditions.

Supplementary Figure 2. Baseline fatty acid (FA) accumulation in wild-type Rhodococcus opacus PD630 and knockout strains ΔLPD07014, ΔLPD07015, and ΔLPD07016 under standard fermentation conditions.