# Aromatic dimer dehydrogenases from *Novosphingobium aromaticivorans* reduce aromatic diketones

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**Running Title: Reduction of aromatic diketones**

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# Abstract

Lignin is a potential source of valuable aromatics, but its chemical depolymerization results in a heterogeneous mixture of products. Microbes could valorize depolymerized lignin by converting multiple aromatic substrates into a one or a small number of products. In this study, we describe the ability of *Novosphingobium aromaticivorans* to metabolize 1-(4-hydroxy-3-methoxyphenyl)-1,2-propanedione (G-diketone), an aromatic Hibbert diketone which is produced during formic acid-catalyzed lignin depolymerization. By assaying genome-wide transcript levels from *N. aromaticivorans* during growth on G-diketone, 2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (GP-1) and other chemically-related aromatics, we hypothesized that the Lig dehydrogenases, previously characterized as oxidizing β-O-4 linkages in aromatic dimers, were involved in G-diketone metabolism by *N. aromaticivorans*. Using purified *N. aromaticivorans* Lig dehydrogenases, we found that LigL, LigN, and LigD each reduced G-diketone *in vitro* but with different substrate specificities and rates. All three Lig dehydrogenases reduced the Cα ketone of the G-diketone side-chain, while LigL, but not LigN or LigD, reduced the Cα ketone when provided GP-1 as a substrate. The newly identified activity of these Lig dehydrogenases expands the potential range of substrates utilized by *N. aromaticivorans* beyond what has been previously recognized. This is beneficial both for metabolizing a wide range of natural and non-native depolymerized lignin substrates and for engineering enzymes that are active with a broader range of aromatic compounds.

**Importance**

Lignin is a major plant polymer composed of aromatic units that have value as chemicals. However, the structure and composition of lignin has made it difficult to use this polymer as a renewable source of industrial chemicals. Bacteria like *Novosphingobium aromaticivorans* have the potential to make chemicals from lignin because of their ability to convert a variety of lignin-derived aromatics into valuable products. In this work, we report a newly discovered activity of previously characterized dehydrogenase enzymes with a chemically-modified byproduct of lignin depolymerization. We propose that the activity of *N. aromaticivorans* enzymes with both native lignin aromatics and those produced by chemical depolymerization will expand opportunities for producing industrial chemicals from the heterogenous components of this abundant plant polymer.

# Introduction

It is estimated that approximately 30% of organic carbon in the biosphere is comprised of lignin (1). Although lignin is abundant, the heterogeneous structure and chemical composition of this aromatic polymer has prevented its widespread use as a source of products for industrial applications (2). Currently, only a small fraction of available lignin is converted into chemicals, leaving the majority of the material either burned for energy or unused as a renewable source of products (3). Overcoming the inherent barriers to valorizing lignin would provide a renewable source for many chemicals currently produced from non-renewable resources (4). We seek to understand bacterial aromatic metabolism in order to biologically convert deconstructed lignin into valuable chemical products.

Guaiacyl (G)- and syringyl (S)-phenylpropanoids are the most abundant monoaromatics in the lignin polymer, with hydroxylbenzoyl (H)-aromatics as common pendent groups (5). Consequently, all existing chemical methods to depolymerize lignin produce mixtures of G-, S- and H-aromatic monomers, along with dimers or oligomers containing combinations of these aromatic units (6). The heterogeneity of depolymerized lignin presents a challenge to the production of single lignin-derived products, but this could potentially be achieved using microbes to funnel diverse lignin-derived aromatic compounds through a central metabolic pathway (7, 8). We study the ability of *Novosphingobium aromaticivorans* DSM12444 (formerly *Sphingomonas aromaticivorans* F199 (9)), an Alphaproteobacterium capable of metabolizing a diverse array of aromatic compounds, to convert depolymerized lignin aromatics into potentially valuable products (10). *N. aromaticivorans* is genetically tractable and degrades many aromatic compounds completely and quickly, making it an excellent organism for studying the metabolism of lignin-derived products (11, 12).

In this study, we investigate how *N. aromaticivorans* metabolizes an aromatic G-diketone (1-(4-hydroxy-3-methoxyphenyl)-1,2-propane dione) that is a chemical byproduct of both a formic acid-catalyzed lignin depolymerization process (13) and dilute acid hydrolysis of several potential lignocellulosic crops (14). Previous work has shown that *N. aromaticivorans* grew on this G-diketone, and that an engineered strain transformed it to 2-pyrone-4-6-dicarboxylic acid (PDC), a potentially valuable lignin-derived product (10). However, the enzymes that participate in G-diketone metabolism have yet to be identified. To identify enzymes and pathways that contribute to metabolism of the G-diketone, we measured global transcript patterns during growth on the G-diketone to other G-type monoaromatics. Based on these data, we hypothesized that the enzymes LigLNDO*,* which encode pyridine nucleotide-dependent dehydrogenases that initiate degradation of β-O-4 linked aromatic dimers in the closely related *Sphingobium* sp. SYK-6 (15), begin the process of metabolizing the G-diketone by reducing one or both of its ketone groups. To test this hypothesis, we purified LigL, LigN, and LigD and monitored the reduction of the G-diketone *in vitro* using NADH as a cofactor. The results of these experiments reveal how G-diketone and its metabolic byproducts enter the known aromatic metabolism pathways of *N. aromaticivorans*, demonstrate an alternative reductive function for dehydrogenases previously proposed to oxidize aromatic β-O-4 linked dimers, and illustrate the potential for microbial conversion of non-native products of chemical lignin depolymerization as a renewable source of valuable chemicals.

# Methods

## Analyzing growth with and metabolism of G-diketone

﻿ *N. aromaticivorans* cultures were grown aerobically in Standard Mineral Broth (SMB; DSMZ Medium 1185) without tryptone or yeast extract (16), supplemented with either glucose, an aromatic substrate, or both, shaken at 200 rpm and incubated at 30 °C. We measured cell density using a Klett-Summerson photoelectric colorimeter with a red filter; one Klett unit (KU) is equivalent to 8x106 CFU/mL for *N. aromaticivorans* (11).

To assay metabolism of the G-diketone, cultures of *N. aromaticivorans* were grown overnight with 1 g/L D-glucose *(Sigma-Aldrich, St. Louis, MO, USA)* before adding an equal volume of SMB containing 1 g/L glucose and incubating for one hour. We then harvested 2 ml of the culture (5000 rpm, 5 min, room temperature) and resuspended the cells into 0.5 mL fresh SMB with no added carbon source. The resuspended cells were inoculated into 50 mL containing either 1g/L glucose or 0.5 g/L glucose plus an amount of G-diketone equivalent to the theoretical chemical oxygen demand (COD) of 0.5 g/L glucose (0.418 g/L G-diketone). 1-mL samples of these cultures were collected as a function of time and harvested (5000 rpm, 5 min, 4 °C). The supernatants of these samples were filtered through 0.22 μm nylon syringe tip filters *(Fisher Scientific, Hampton, NH, USA)*, and analyzed on an Agilent 1260 Infinity HPLC (*Agilent Technologies, Inc., Palo Alto, CA, USA*). The mobile phase was a binary gradient consisting of water and 0.1% formic acid in a 2:1 v/v mixture of acetonitrile and methanol (10).

## RNA-Seq culture conditions

The strain used for RNA analysis was *N. aromaticivorans* DSM12444 containing a deletion of the gene *sacB* (SARO\_RS09410, Saro\_1879), which conveys sucrose sensitivity (11). All cultures were grown in SMB with 0.5 g/L of D-glucose plus an indicated aromatic, as cultures grown on some aromatic compounds as the sole organic substrate produced insufficient biomass for RNA analysis. Amounts of aromatic substrate in the culture were normalized to have a theoretical COD equivalent to 0.5 g/L. The aromatic substrates were dissolved in dimethyl sulfoxide (DMSO) (5 μL per 10 mL of the final culture) prior to addition to the media. Cells grown in the presence of 1 g/L of glucose were used as the control culture for RNA analysis. Aromatic substrates added to cultures were protocatechuic acid (PCA), vanillic acid, vanillin, ferulic acid *(Sigma-Aldrich, St. Louis, MO, USA),* 2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (GP-1) *(Key Organics, Camelford, UK)*, and G-diketone, synthesized as previously described (10).

## RNA preparation

A 25-mL overnight culture grown in the presence of SMB plus glucose provided the inoculum for all cultures used for RNA extraction. Before shifting cells to media containing an aromatic substrate, we added 25 mL of SMB plus glucose and waited 40 min to allows cells to achieve growth. At this time, 2-mL aliquots of this culture were harvested (6000 rpm, 5 min, 21 °C), washed in 1 mL of SMB with no added carbon source, and resuspended in 100 μL of SMB with no added carbon source. This resuspended material was split into 25-mL triplicate cultures containing either glucose and an aromatic compound or only glucose as the sole carbon source at concentrations described above. These cultures were incubated overnight and then amended with 25 mL of fresh identical media to ensure logarithmic growth. After 40 min, cell growth was terminated by adding 5.7 mL of cold 95% ethanol + 5% acid phenol:chloroform to the entire 50-mL culture. Cells from each culture were harvested (6000 rpm, 12 min, 4 °C), the pellets were flash-frozen in ethanol and dry ice, and the supernatant from each culture was filtered and stored at -80 °C for subsequent HPLC analysis (see above).

Thawed cell pellets were lysed using an SDS/EDTA-based buffer at 65 °C (11). Genomic DNA was removed and RNA purified using the Qiagen RNEasy Kit *(Qiagen, Hilden, Germany)*. An on-column DNAse digestion *(Qiagen, Hilden, Germany)* was performed to remove remaining DNA. RNA was eluted in 50 μL nuclease-free water *(Life Technologies, Carlsbad, CA, USA)* and stored at -80 °C until preparation for sequencing. RNA yield was assessed using a Qubit Broad-Range RNA assay *(Life Technologies, Carlsbad, CA, USA)*.

## RNA sequencing and bioinformatics

RNA-Seq library preparation and sequencing was performed at the Department of Energy Joint Genome Institute (*Berkeley, CA, USA*). Libraries were created using the Illumina TruSeq Stranded Total RNA kit *(Illumina, San Diego, CA, USA)* following the standard protocol, which included ribosomal RNA depletion using the RiboZero bacterial kit *(Illumina, San Diego, CA, USA).* Four RNA-seq libraries were sequenced per lane on an Illumina NextSeq *(Illumina, San Diego, CA, USA)* in 2x151 reads using the manufacturer’s standard protocol.

After sequence analysis, the paired-end FASTQ files were split into those containing forward and reverse reads, and forward read files were retained for further analysis. Sequence reads were trimmed using Trimmomatic version 0.3 (17) with the default settings except for a HEADCROP of 5, LEADING of 3, TRAILING of 3, SLIDINGWINDOW of 3:30, and MINLEN of 36. After trimming, the sequence reads were aligned to the *N. aromaticivorans* genome sequence (GenBank accession NC\_007794.1) using Bowtie2 version 2.2.2 (18) with default settings except the number of mismatches was set to 1. Aligned sequence reads were mapped to gene locations using HTSeq version 0.6.0 (19) with default settings except for the “reverse” strandedness argument was used. The software edgeR version 3.26.8 (20) was used to identify significantly differentially expressed genes from pairwise analyses, using a Benjamini and Hochberg false discovery rate (FDR) less than 0.05 as a significance threshold (21). Raw sequencing reads were normalized using the reads per kilobase per million mapped reads (RPKM).

## Construction of N. aromaticivorans mutants

Strains containing individual in-frame deletions of *ligL, ligN, ligD*, and *ligO* (SARO\_RS09390, SARO\_RS03965, SARO\_RS01025, and SARO\_RS03960) were generated in a *sacB* mutant strain using previously described plasmids and methods (22). Briefly, strains of the *Escherichia coli* WM3064 containing non-replicating pAK405 vectors with approximately 450 base pairs of DNA flanking each desired gene deletion were conjugated on plates in a 1:5 ratio with the *N. aromaticivorans sacB* recipient(23)*.* Conjugates were selected by plating onto LB with kanamycin. Conjugates were grown overnight in 5-mL cultures of LB and plated onto LB with streptomycin to select for strains containing double crossover events. Mutants with desired gene deletions were confirmed via patching onto LB plus streptomycin and LB plus kanamycin, as well as via PCR of genomic DNA with gene-specific primers. Growth of confirmed deletion mutants and the *sacB* parent strain was tested in 25-mL triplicate flask cultures containing 1 mmol/L glucose + 1 mmol/L G-diketone in SMB + 0.05% DMSO, as well as additional cultures grown in 2 mmol/L glucose as a control.

## In vitro enzyme assays

The genes *ligL* (SARO\_RS09390)*, ligN* (SARO\_RS03965)*,* and *ligD* (SARO\_RS01025) were amplified from *N. aromaticivorans* DSM 12444 and separately cloned into plasmid pVP302K (24) containing an N-terminal His8 tag. The expression plasmids were transformed into *E. coli* B834 containing pRARE2 plasmid (*Novagen, Gibbston, NJ, USA*). Proteins expression was induced by growing the *E. coli* strains at 25 °C for 25 h in ZYM-5052 autoinduction medium containing kanamycin and chloramphenicol. Cells were harvested by centrifugation. The resulting pellet was resuspended in lysis buffer (50 mM NaH2PO4, 100 mM NaCl, 5 mM imidazole, 10% glycerol, 0.5 mM TCEP, and 1% TritonX-100) and lysed by sonication. Following centrifugation of the lysates, the supernatant was filtered through a 0.22-μm, 33-mm diameter, polyethersulfone filter *(MilliporeSigma, Burlington, MA, USA)* and passed through a gravity column packed with Ni2+-NTA resin (*Qiagen, Hilden, Germany*), washed with wash buffer (50 mM NaH2PO4, 200 mM NaCl, 25 mM imidazole, and 0.5 mM TCEP) and eluted in elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, and 0.5mM TCEP). The eluted proteins were concentrated using an Amicon® Ultra-15 centrifugal filter units (*MilliporeSigma, Burlington, MA, USA*) and dialyzed into dialysis buffer (50 mM NaH2PO4, 100 mM NaCl, and 0.5 mM TCEP). Protein concentrations were determined using the Bradford method.

These purified enzymes were used for *in vitro* enzymeassays using G-diketone as a potential aromatic substrate and with or without NADH *(Sigma-Aldrich, St. Louis, MO, USA)* as a cofactor, in a buffer containing Tris-HCl (pH 8) and NaCl *(Sigma-Aldrich, St. Louis, MO, USA)*. An additional control was run with no enzyme added to assess spontaneous degradation of the G-diketone. In some cases, concentrations of the G-diketone were measured at 0, 1, 2, 3, and 24 hours using HPLC. We tested for the presence of additional reaction products by GC/MS (GC model 7890A, MS Model 5975C) *(Agilent Technologies, Inc., Palo Alto, CA, USA*) using material from the 24-hour timepoint. Samples were acidified with HCl and extracted with ethyl acetate three times. The collected organic phase was evaporated and trimethylsilyl derivatized.

We also performed spectrophotometric NADH oxidation/NAD+ reduction assays using LigL, LigN, and LigD incubated in the presence of G-diketone, GP-1, and guaiacyl-glycerol-β-guaiacyl ether (GGE), and threo-1-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol (threo-GD) (*BioCrick Co. Ltd., Chengdu, Sichuan, China*). Assay conditions were set as previously described, with NADH/NAD+ added immediately prior to measurement of optical density at 340 nm on an Olis DW-2000 spectrophotometer *(OLIS, Inc., Athens, GA, USA).* An assay with no cofactor was used as the reference for each run. Substrate concentrations were varied to determine the kinetic parameters of each enzyme.

# Results

## Extracellular products are transiently accumulated during G-diketone utilization

We previously demonstrated that *N. aromaticivorans* can grow on either G-diketone or 1-(4-hydroxy-3,4-dimethoxyphenyl)-1,2-propane dione (S-diketone), and that these compounds were each converted to PDC when an engineered strain was grown in the presence of these compounds (10). This finding predicted that both of these diketones were metabolized through a pathway that included PDC as an intermediate. However, the pathway and enzymes involved in aromatic diketone degradation have yet to be described. We focused this study on dissecting the metabolism of G-diketone, which was the diketone with the highest conversion yield to PDC (10).

Although *N. aromaticivorans* will grow if G-diketone is provided as the sole carbon source (10), we grew cells in media containing both this aromatic compound and glucose in order to obtain sufficient biomass for our studies. Under these conditions, HPLC analyses of the extracellular fractions showed a time-dependent disappearance of G-diketone from the culture media, as expected if it is imported and metabolized by *N. aromaticivorans* (Figure 1, Figure S1). HPLC analysis of extracellular materials also detected the time-dependent accumulation and subsequent loss of a UV-absorbing compound in the media of cells grown in the presence of G-diketone (Figure 1), suggesting that it was an aromatic compound. One interpretation of these observations is that the UV-absorbing compound which transiently accumulated when cells are grown in the presence of glucose and G-diketone was an intermediate in the metabolism of this aromatic compound. To test this hypothesis, supernatant from these cultures was collected, aromatic compounds recovered by liquid-liquid extraction, and then derivatized for analysis by GC-MS. The GC trace showed the transient accumulation of a compound concomitant with the consumption of G-diketone (Figure SX), similar to what we observed by HPLC (Figure 1). The observed molecular mass of the derivatized material suggested that it could be a byproduct of G-diketone in which one of the ketones was reduced to a hydroxyl group. The agreement of the MS spectrum of the derivatized product with that of GP-1 (Figure SX), the byproduct of G-diketone with the Cβ ketone reduced to a hydroxyl group, validated the hypothesis. However, in the absence of a pure sample of 1-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-2-one (GP-2), another byproduct of G-diketone with the Cα ketone reduced to a hydroxyl group, we were unable to unequivocally determine whether the observed extracellular product was a single compound (GP-1 or GP-2) or the combination of both compounds. Regardless, this finding predicts that *N. aromaticivorans* contains enzymes for reducing one or both ketone moieties in G-diketone.

## Genome scale changes in transcript abundance during growth in the presence of G-diketone and other G-type aromatics

To identify candidates for gene products involved in metabolism of the G-diketone, we compared transcript abundance between *N. aromaticivorans* cells growing in the presence of the G-diketone, other G-type aromatics (PCA, vanillic acid, vanillin, GP-1, and ferulic acid), or glucose (as a non-aromatic carbon source). We found that the abundance of several hundred transcripts was altered in cells growing in the presence of the G-diketone and the other G-type aromatics (Base G in Figure 2) when compared to cells grown in the presence of glucose as a sole organic carbon course. Additional differences in transcript abundance were observed between cultures grown in the presence of GP-1 and the other aromatics, and the also between cultures grown on G-diketone compared to the other G compounds (Figure 2).

Since the number of transcripts with differential abundance in these comparisons was in the hundreds (Figure 2), we focused on genes encoding enzymes known to participate in aromatic metabolism by *N. aromaticivorans* (Figure 4). These differently expressed transcripts included those from genes encoding NAD-dependent dehydrogenases (*ligD, ligO, ligL, ligN*) (15, 24), glutathione S-transferases (*ligE, ligF, baeAB*) (25), and glutathione lyases (*NaGSTNu*) (11) that are known or predicted to be involved in breaking the β-O-4 aromatic linkage in lignin. Most of these *lig* genes are not co-located on the genome (Figure S2), so the increase in abundance of these transcripts likely results from an increase in expression of several transcription units. Therefore, the above genes encoding enzymes that act on aromatic dimers are also grouped as genes with significant changes in expression in the presence of the G-diketone (Figure 3).

We also analyzed supernatants of these same cultures via HPLC for the presence of aromatic compounds. Cultures grown in the presence of glucose and either PCA, vanillic acid, vanillin, and ferulic acid contained significant amounts of the aromatic carbon source provided and lower concentrations of known intermediates in their metabolism (Supplemental Dataset S1). Both vanillic acid and vanillin were found at low levels in the media of cells grown on GP-1 (Dataset S1). Vanillic acid and vanillin are not present at detectable levels in GP-1 (Table S1), so the presence of these two aromatics in the media of cells grown on GP-1 suggests they are byproducts of cellular metabolism of this aromatic compound.

*In vitro activity of Lig dehydrogenases with G-diketone*

The above result identifies genes encoding Lig dehydrogenases that have previously been shown to oxidize β-O-4 linked aromatic dimers as candidates in a pathway for utilization of G-diketone and GP-1. To investigate a potential role of *N. aromaticivorans* Lig dehydrogenases with G-diketone, we purified recombinant Lig dehydrogenases and tested their activity *in vitro.* Since homologues of these enzymes have been studied for their NAD-dependent dehydrogenase activity on β-O-4 linked aromatic dimers (24), we first measured the kinetic parameters of these recombinant Lig proteins via spectrophotometric NADH oxidation/NAD+ reduction assays with guaiacyl-glycerol-β-guaiacyl ether (GGE), a β-O-4 linked aromatic dimer as substrate (Figure 4). Purified recombinant versions of LigL, LigN, and LigD showed relatively similar turnover frequencies (Kcat) and Michaelis-Menten half-saturation constants (Km) when incubated with GGE and NAD+, as expected given previous reports of their homologues from *Sphingobium* SYK-6 with this substrate (26). We were not able to obtain a recombinant LigO protein that was active when incubated with GGE, so no further assays were performed with this enzyme.

When testing recombinant LigL, LigN and LigO enzymes for activity with G-diketone *in vitro* we found that this aromatic compound was reduced in a time-dependent manner in the presence of NADH (Figure 5). The time-dependent reduction of G-diketone required the presence of NADH (Figure 5), suggesting that this pyridine nucleotide is a cofactor for this activity with all three dehydrogenases. To better understand the role of NADH in activity of these dehydrogenases with G-diketone as an aromatic substrate, we analyzed the aromatic products of the *in vitro* reactions by GC-MS (Figure 6). In reactions with each of the three dehydrogenase enzymes with the G-diketone and NADH, the G-diketone was undetectable after 24 hours and a reduced aromatic that we identified as GP-2 was produced. This result demonstrates that LigL, LigN and LigO each have the ability to reduce one of the ketones of G-diketone when NADH is provided as a source of reducing power.

When GP-1 was incubated with LigL and NADH, we observed two additional aromatic products (Figure 6). Using a commercially available compound, we identified one of these products as threo-1-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol (GD). The other product had the same mass as threo-GD and we propose that it is the erythro- form of GD (Figure 6, Panel X). In contrast, when GP-1 is incubated with NADH and either active forms of LigN and LigD, neither GP-1 or NADH was consumed and no aromatic products were observed (Figure 6). Thus, it appears that LigL is the only one of these three dehydrogenases that is able to reduce GP-1.

The requirement for NADH in order to see reduction of G-diketone by these Lig dehydrogenases predicts that is a source of reducing power for this reaction. We also used the oxidation of NADH as a reporter to test this hypothesis and determine kinetic constants for each of the three recombinant Lig dehydrogenases with either G-diketone or GP-1 as an aromatic substrate. These experiments revealed that the apparent Km values for G-diketone with each Lig dehydrogenase were of the same order of magnitude when LigL, LigN and LigO were individually incubated in the presence of the G-diketone and NADH, indicating similar enzyme affinity of each enzyme for this substrate (Figure 4). In addition, the apparent Km values of each of the three dehydrogenases for G-diketone and GGE (when assayed in the presence of NAD+ as an electron acceptor) were comparable, indicating that they have similar affinities for these two aromatic substrates (Figure 4). However, the Kcat value for LigL when using G-diketone was consistently higher than that observed with GGE, while LigN and LigD had significantly lower Kcat values for G-diketone than GGE (Figure 4). This finding indicates that LigL acts faster on the G-diketone than GGE compared to LigN and LigD. We attempted to measure kinetic parameters of the LigL with GP-1 and NADH, but absorbance of a putative reaction product at 340 nm interfered with our ability to monitor NADH oxidation. When LigL was incubated with threo-GD, reduction of NAD+ was observed, although we were unable to identify a product (Figure 4). No reduction of NAD+ was observed when LigN or LigD were incubated with threo-GD (Figure 4).

*Genetic analysis of the role of Lig aromatic dehydrogenases in G-diketone utilization*

The results of the above *in vitro* enzyme assays indicate that LigL, LigN and LigO each reduce G-diketone. If each of the Lig dehydrogenases can contribute to G-diketone metabolism *in vivo*, it predicts that loss of any single Lig dehydrogenase would not have a significant impact on the growth or the production of microbial biomass of *N. aromaticivorans* in the presence ofthis aromatic compound. To test this hypothesis, we created strains with individual in-frame deletions of *ligL*, *ligN*, *ligD*,and *ligO* and tested their growth in media containing either glucose alone or glucose and G-diketone. Of the four individual mutants, none showed any significant defects in either growth rate or total biomass produced when grown on glucose as the sole carbon source or on a combination of glucose and the G-diketone (Figure S3). This data shows that none of these individual dehydrogenases is required for growth in media containing glucose and G-diketone and it predicts that they can each support sufficient reduction of this aromatic to support wild type growth rates*.*

# Discussion

Microbial funneling of the products of depolymerized lignin to valuable compounds has the potential to overcome an existing challenge caused by the heterogeneous chemical composition of this polymer. Unlike many abiotic methods, microbes can simultaneously convert mixtures of aromatic monomers to one or a few desired products, either naturally or when engineered (10, 12). Several bacteria are potential chassis organisms for microbial funneling of depolymerized lignin. Sphingomonad bacteria such as *N. aromaticivorans* and *Sphingobium* SYK-6 have the native ability to cleave β-O-4 ether linkages in lignin-derived aromatic dimers (24) and can be engineered to metabolize a wide range of substrates to produce a valuable compound such as PDC(10, 12). Similarly, the Gammaproteobacteria *Pseudomonas putida* can either naturally or be engineered to produce valuable chemicals from one or more aromatic substrates (27–29).Other potential bacterial products of depolymerized lignin include *cis-cis* muconic acid, lipids, or polyhydroxyalkonates (30–34).

The success of microbial funneling is likely dependent on both the organism and the lignin depolymerization method. Some microbes may be better suited to funnel both native and chemically-modified products of lignin depolymerization, either before or after the addition of metabolic functions from another host. In this study, we examined how *N. aromaticivorans* consumes G-diketone, a phenylpropanone that is abundant in the products formed from a formic acid-induced lignin depolymerization method (13) and is detected in the products of dilute acid hydrolysis of several potential lignocellulosic biofuel feedstocks (14). This G-diketone belongs to a group of compounds known as Hibbert ketones (14), for which information on microbial degradation pathways is lacking. Below, we discuss the implications of our findings on the enzymes involved in the process of G-diketone degradation by *N. aromaticivorans*.

Previously characterized Lig dehydrogenases reduce G-diketone*.*

Based on genome-wide transcript analysis of cells grown in the presence of G-diketone, we hypothesized that the known aromatic dehydrogenases LigLNDOplayed a role in G-diketone metabolism, possibly by reducing one or both ketones on the aromatic sidechain. To test this hypothesis, we compared enzyme activity for LigL, LigN, and LigD incubated with the G-diketone or GGE, a model aromatic dimer. *In vitro* enzyme assays confirmed that LigL, LigN, and LigD were all able to reduce the Cα ketone of G-diketone in a reaction that required NADH as a source of reductant to produce GP-2. Each of these recombinant Lig dehydrogenases bound G-diketone and GGE with a similar affinity, since the Michaelis-Menten constants for the monoaromatic and dimeric substrates were comparable under identical reaction conditions (Figure 4). This predicts that the presence or absence of a second aromatic ring does not make a major contribution to binding of this substrate. Previous analysis of a Lig β-etherase that participates in GGE metabolism has shown that this enzyme is active with both β-O-4 linked aromatic dimers and monomers that contain different side chains (24). These and our observations indicate that Lig enzymes analyzed in the past may have a broader role in the metabolism or biologically derived aromatics and those that are produced during chemical treatment of lignin and other aromatic-containing substrates. Indeed, our experiments show that *N. aromaticivorans* LigL, LigN and LigO are each able to oxidize the side chain of an aromatic dimer like GGE, reduce the Cα-side chain ketone in G-diketone to generate GP-2, and that LigL reduces both of the Cα- and Cβ-ketones to produce GD (Figure 6).

In this regard, we found that LigN and LigD had higher turnover frequencies when oxidizing the Cα bond in the aromatic dimer GGE compared to reducing the Cα ketone in the G-diketone (Figure 4). In contrast, LigL had a higher turnover frequency when reducing the Cα ketone in the G-diketone than when oxidizing the Cα position of GGE. This predicts there might be some active site differences in these three aromatic dehydrogenases that impact their ability to oxidize or reduce individual substrates. Additionally, we found that LigL was able to reduce the Cα ketone to form GD when provided GP-1 as a substrate (Figure 6), while LigN and LigD could not reduce GP-1 (Figure 6). This suggests that LigL catalyzes another step in G-diketone degradation, resulting in a fully reduced side chain that is a substrate for subsequent cleavage to produce vanillin (is this correct, but we also say this in the next paragraph…. Maybe just end this sentence after fully reduced side chain and save the later steps for the model below?) (Figure 7?). LigL was also able to catalyze the reverse reaction, oxidizing threo-GD, while LigN and LigD could not. Hibbert ketones such as GP-2 and GP-1 are known to spontaneously interconvert, so it is possible that, under cellular conditions, GP-2 can isomerize to GP-1 (14). Deletion of *ligL* did not result in a growth defect on the G-diketone, so it is possible that another as of yet unknown enzyme is capable of reducing GP-2 to GD. Each of the Lig dehydrogenases oxidizes a different stereoisomer of GGE (26), so it is likely that stereochemistry may play an important role in enzymatic processing of the G-diketone sidechain. However, sources of erythro-GD are needed to assess the relative activity of LigD, LigL, and LigN with this compound.

In vivo degradation of the G-diketone

Our in vitro enzyme assays predicted that G-diketone degradation is initialized by the NADH-dependent reduction of the Cα ketone, producing GP-2. When *N. aromaticivorans* was grown in the presence of the G-diketone + glucose, we observed the transient extracellular accumulation of a product with the same molecular weight as either GP-1 or GP-2 (Figure 1), but were unable to separate the two predicted compounds in this material to determine which ketone was reduced.

Based on our findings, we can generate a model for metabolism of G-diketone by *N. aromaticivorans* (Figure 7)*.* This model predicts that the Cα ketone of the G-diketone is reduced by one of several redundant Lig dehydrogenases to GP-2, which can then isomerize to GP-1. GP-1 is further reduced by LigL to GD. We further propose that the sidechain of GD is cleaved by an as of yet uncharacterized enzyme(s), producing an unknown two-carbon product and vanillin, which is oxidized by LigV in a previously proposed vanillin degradation pathway (35) (Figure 7). Transcripts derived from *N. aromaticivorans* ligV, which encodes a homologue of a Sphingobium SYK-6 enzyme responsible for oxidizing vanillin to vanillic acid, were more abundant during growth on both the G-diketone and GP-1 (Figure 4) (35), suggesting that vanillin is an intermediate in the G-diketone pathway. Unfortunately, our G-diketone preparations contain trace amounts of vanillin, making it difficult to assess the source of low extracellular levels of this compound in cells grown in the presence of G-diketone (Table S1). However, the transient extracellular accumulation of vanillin and vanillic acid when cells are grown in the presence of GP-1 (Dataset S1) supports the predicted pathway for *N. aromaticivorans* G-diketone metabolism*.*

*Substrate specificity of Lig pathway enzymes*

When taken together, this work expands our knowledge of the ability of *N. aromaticivorans* to metabolize and funnel a diversity of aromatic compounds into a common pathway. While this work focused on metabolism of G-diketone, but previous studies have shown that cells can grow on S-diketone (10) and that several Lig enzymes are active with S-type aromatic substrates as well (24, 36). We propose that the use of multi-functional enzymes may provide *N. aromaticivorans* with the flexibility to consume a broad mix of monomeric and oligomeric aromatic substrates.

There are other examples of *N. aromaticivorans* enzymes that can act on both aromatic monomers and dimers. NaLigF2 is a β-etherase that cleaves aromatic dimers in the presence of glutathione (24). However, based on fitness analysis of a mutant library of *N. aromaticivorans*, this same gene product was proposed to be a subunit of a DesCD isomerase that converts 4-carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate (CHMOD) to (4 E)-oxalomesaconate (OMA) during syringic acid degradation (22). As predicted, mutations that inactivate *desD* resulted in a loss of growth on syringic acid and accumulation of CHMOD, and *in vitro* enzyme assays showed that purified DesCD produced OMA (22). Additionally, the syringate o-demethylase DesA can also demethylate vanillic acid, albeit at a slower rate, and a second ring-opening dioxygenase, LigAB2, has been identified in *N. aromaticivorans* (12). Thus, it is possible that broad substrate specificity may be an underappreciated and more common feature of *N. aromaticivorans* aromatic metabolism.

The presence of overlapping, multi-functional, aromatic-degrading enzymes may allow *N. aromaticivorans* to adapt to, metabolize, and persist in the presence of a wide set of aromatic substrates that it might find in nature. This enzymatic flexibility is also potentially advantageous from an industrial production standpoint, since *N. aromaticivorans* could be well suited for metabolism of both naturally occurring aromatics and those that are chemical byproducts of lignin or other aromatic substrates. Relatively broad substrate specificity of *N. aromaticivorans* enzymes may also provide scaffolds for improvement in catalytic rate or utilization of aromatic substrates.

In conclusion, we coupled metabolite analyses, transcriptomics and enzyme assays to predict and identify proteins responsible for the degradation of the G-diketone in *N. aromaticiovorans.* These studies showed that three Lig dehydrogenases previously described for a role in oxidation of a Cα carbon in β -O-4 linked aromatic dimers reduce the Cα ketone as a first step in the metabolism of G-diketone. Based on this and other published studies, we propose that redundant multi-functional enzymes are a key feature of *N. aromaticivorans’* aromatic metabolism, allowing it to degrade diverse aromatic compounds, including ones found in nature and those generated as a byproduct of chemical deconstruction of lignin. This finding expands our understanding of microbial aromatic metabolism, and it furthers our ability to identify and engineer a microbial strain capable of valorizing lignin or chemical products of its deconstruction as part of biorefinery pipeline.

# Data availability

Raw sequencing reads are available through the DOE Joint Genome Institute Genome Portal with Project ID 1233250 (<https://genome.jgi.doe.gov/portal/Novarocriptomics_FD/Novarocriptomics_FD.info.html>

). Code and processed data files used in this project are available via the GitHub repo <https://github.com/alexlinz/Guaiacyl_degradation>

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# References

1. Boerjan W, Ralph J, Baucher M. 2003. Lignin Biosynthesis. Annu Rev Plant Biol 54:519–546.

2. Cao Y, Chen SS, Zhang S, Ok YS, Matsagar BM, Wu KC-W, Tsang DCW. 2019. Advances in lignin valorization towards bio-based chemicals and fuels: Lignin biorefinery. Bioresour Technol 291:121878.

3. Ragauskas AJ, Beckham GT, Biddy MJ, Chandra R, Chen F, Davis MF, Davison BH, Dixon RA, Gilna P, Keller M, Langan P, Naskar AK, Saddler JN, Tschaplinski TJ, Tuskan GA, Wyman CE. 2014. Lignin valorization: Improving lignin processing in the biorefinery. Science (80- ). American Association for the Advancement of Science.

4. Becker J, Wittmann C. 2019. A field of dreams: Lignin valorization into chemicals, materials, fuels, and health-care products. Biotechnol Adv.

5. Ralph J, Lapierre C, Boerjan W. 2019. Lignin structure and its engineering. Curr Opin Biotechnol 56:240–249.

6. Das A, Rahimi A, Ulbrich A, Alherech M, Motagamwala AH, Bhalla A, da Costa Sousa L, Balan V, Dumesic JA, Hegg EL, Dale BE, Ralph J, Coon JJ, Stahl SS. 2018. Lignin Conversion to Low-Molecular-Weight Aromatics via an Aerobic Oxidation-Hydrolysis Sequence: Comparison of Different Lignin Sources. ACS Sustain Chem Eng 6:3367–3374.

7. Kamimura N, Sakamoto S, Mitsuda N, Masai E, Kajita S. 2019. Advances in microbial lignin degradation and its applications. Curr Opin Biotechnol 56:179–186.

8. Davis K, Moon TS. 2020. Tailoring microbes to upgrade lignin. Curr Opin Chem Biol 59:23–29.

9. Romine MF, Stillwell LC, Wong KK, Thurston SJ, Sisk EC, Sensen C, Gaasterland T, Fredrickson JK, Saffer JD. 1999. Complete sequence of a 184-kilobase catabolic plasmid from Sphingomonas aromaticivorans F199. J Bacteriol 181:1585–1602.

10. Perez JM, Kontur WS, Alherech M, Coplien J, Karlen SD, Stahl SS, Donohue TJ, Noguera DR. 2019. Funneling aromatic products of chemically depolymerized lignin into 2-pyrone-4-6-dicarboxylic acid with: Novosphingobium aromaticivorans. Green Chem 21:1340–1350.

11. Kontur WS, Bingman CA, Olmsted CN, Wassarman DR, Ulbrich A, Gall DL, Smith RW, Yusko LM, Fox BG, Noguera DR, Coon JJ, Donohue TJ. 2018. Novosphingobium aromaticivorans uses a Nu-class glutathione S-transferase as a glutathione lyase in breaking the -aryl ether bond of lignin. J Biol Chem 293:4955–4968.

12. Perez JM, Kontur WS, Gehl C, Gille DM, Ma Y, Niles A V., Umana G, Donohue TJ, Noguera DR. 2021. Redundancy in aromatic O-demethylation and ring opening reactions in Novosphingobium aromaticivorans and their impact in the metabolism of plant derived phenolics . Appl Environ Microbiol 1–23.

13. Rahimi A, Ulbrich A, Coon JJ, Stahl SS. 2014. Formic-acid-induced depolymerization of oxidized lignin to aromatics. Nature 515:249–252.

14. Mitchell VD, Taylor CM, Bauer S. 2014. Comprehensive Analysis of Monomeric Phenolics in Dilute Acid Plant Hydrolysates. Bioenergy Res 7:654–669.

15. Pereira JH, Heins RA, Gall DL, McAndrew RP, Deng K, Holland KC, Donohue TJ, Noguera DR, Simmons BA, Sale KL, Ralph J, Adams PD. 2016. Structural and biochemical characterization of the early and late enzymes in the lignin β-aryl ether cleavage pathway from sphingobium sp. SYK-6. J Biol Chem 291:10228–10238.

16. Stanier RY, Palleroni NJ, Doudoroff M. 1966. The aerobic pseudomonads: a taxonomic study. J Gen Microbiol 43:159–271.

17. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120.

18. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359.

19. Anders S, Pyl PT, Huber W. 2015. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169.

20. Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140.

21. Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B 57:289–300.

22. Cecil JH, Garcia DC, Giannone RJ, Michener JK. 2018. Rapid, Parallel Identification of Catabolism Pathways of Lignin-Derived Aromatic Compounds in Novosphingobium aromaticivorans. Appl Environ Microbiol 84:e01185-18.

23. Kaczmarczyk A, Vorholt JA, Francez-Charlot A. 2012. Markerless gene deletion system for sphingomonads. Appl Environ Microbiol 78:3774–3777.

24. Gall DL, Ralph J, Donohue TJ, Noguera DR. 2014. A Group of Sequence-Related Sphingomonad Enzymes Catalyzes Cleavage of β-Aryl Ether Linkages in Lignin β-Guaiacyl and β-Syringyl Ether Dimers. Environ Sci Technol 48:12454–12463.

25. Kontur WS, Olmsted CN, Yusko LM, Niles A V., Walters KA, Beebe ET, Vander Meulen KA, Karlen SD, Gall DL, Noguera DR, Donohue TJ. 2019. A heterodimeric glutathione S-transferase that stereospecifically breaks lignin’s (R)-aryl ether bond reveals the diversity of bacterial -etherases. J Biol Chem 294:1877–1890.

26. Sato Y, Moriuchi H, Hishiyama S, Otsuka Y, Oshima K, Kasai D, Nakamura M, Ohara S, Katayama Y, Fukuda M, Masai E. 2009. Identification of three alcohol dehydrogenase genes involved in the stereospecific catabolism of arylglycerol-β-aryl ether by Sphingobium sp. strain SYK-6. Appl Environ Microbiol 75:5195–5201.

27. Vardon DR, Franden MA, Johnson CW, Karp EM, Guarnieri MT, Linger JG, Salm MJ, Strathmann TJ, Beckham GT. 2015. Adipic acid production from lignin. Energy Environ Sci 8:617–628.

28. Elmore JR, Dexter GN, Salvachúa D, Martinez-Baird J, Hatmaker EA, Huenemann JD, Klingeman DM, Peabody GL, Peterson DJ, Singer C, Beckham GT, Guss AM. 2021. Production of itaconic acid from alkali pretreated lignin by dynamic two stage bioconversion. Nat Commun 12.

29. Otsuka Y, Nakamura M, Shigehara K, Sugimura K, Masai E, Ohara S, Katayama Y. 2006. Efficient production of 2-pyrone 4,6-dicarboxylic acid as a novel polymer-based material from protocatechuate by microbial function. Appl Microbiol Biotechnol 71:608–614.

30. Johnson CW, Salvachúa D, Khanna P, Smith H, Peterson DJ, Beckham GT. 2016. Enhancing muconic acid production from glucose and lignin-derived aromatic compounds via increased protocatechuate decarboxylase activity. Metab Eng Commun 3:111–119.

31. Xu Z, Lei P, Zhai R, Wen Z, Jin M. 2019. Recent advances in lignin valorization with bacterial cultures: microorganisms, metabolic pathways, and bio-products. Biotechnol Biofuels 12:32.

32. Shields-Menard SA, AmirSadeghi M, Green M, Womack E, Sparks DL, Blake J, Edelmann M, Ding X, Sukhbaatar B, Hernandez R, Donaldson JR, French T. 2017. The effects of model aromatic lignin compounds on growth and lipid accumulation of Rhodococcus rhodochrous. Int Biodeterior Biodegrad.

33. He Y, Li X, Ben H, Xue X, Yang B. 2017. Lipid Production from Dilute Alkali Corn Stover Lignin by Rhodococcus Strains. ACS Sustain Chem Eng 5:2302–2311.

34. Liu ZH, Olson ML, Shinde S, Wang X, Hao N, Yoo CG, Bhagia S, Dunlap JR, Pu Y, Kao KC, Ragauskas AJ, Jin M, Yuan JS. 2017. Synergistic maximization of the carbohydrate output and lignin processability by combinatorial pretreatmentf. Innov Green Process Eng Sustain Energy Environ 2017 - Top Conf 2017 AIChE Annu Meet 2017-October:248–264.

35. Masai E, Yamamoto Y, Inoue T, Takamura K, Hara H, Kasai D, Katayama Y, Fukuda M. 2007. Characterization of ligV essential for catabolism of vanillin by Sphingomonas paucimobilis SYK-6. Biosci Biotechnol Biochem 71:2487–2492.

36. Gall DL, Ralph J, Donohue TJ, Noguera DR. 2017. Biochemical transformation of lignin for deriving valued commodities from lignocellulose. Curr Opin Biotechnol 45:120–126.