

1 **A concerted systems biology analysis of phenol metabolism in *Rhodococcus***
2 ***opacus* PD630**

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26 **Abstract**

27 *Rhodococcus opacus* PD630 metabolizes aromatic substrates and naturally produces branched-
28 chain lipids, which are advantageous traits for lignin valorization. To provide insights into its
29 lignocellulose hydrolysate utilization, we performed ¹³C pathway tracing, ¹³C-pulse-tracing,
30 transcriptional profiling, biomass compositional analysis, and metabolite profiling in conjunction
31 with ¹³C-metabolic flux analysis (¹³C-MFA) of phenol metabolism. We found that 1) phenol is
32 metabolized mainly through the ortho-cleavage pathway; 2) phenol-utilization requires a highly
33 active TCA cycle; 3) NADPH is generated mainly via NADPH-dependent isocitrate
34 dehydrogenase; 4) active cataplerotic fluxes increase plasticity in the TCA cycle; 5)
35 gluconeogenesis occurs partially through the reversed Entner-Doudoroff pathway (EDP). We also
36 found that phenol-fed *R. opacus* PD630 generally has lower sugar phosphate concentrations (e.g.,
37 fructose 1,6-bisphosphatase) compared to metabolite pools in ¹³C-glucose-fed *Escherichia coli*
38 (set as internal standards), while its TCA metabolites (e.g., malate, succinate, and α -ketoglutarate)
39 accumulate intracellularly with measurable succinate secretion. In addition, we found that phenol
40 utilization was inhibited by benzoate, while catabolite repressions by other tested carbon substrates
41 (e.g., glucose and acetate) were absent in *R. opacus* PD630. Three adaptively-evolved strains
42 display very different growth rates when fed with phenol as a sole carbon source, but they maintain
43 a conserved flux network. These findings improve our understanding of *R. opacus*' metabolism
44 for future lignin valorization.

45

46 **Key words:** ¹³C-MFA, ¹³C pulse-tracing, Entner-Doudoroff pathway, Gluconeogenesis, Lignin

47

48 **Introduction**

49 Engineering bacterial cells to utilize lignocellulosic biomass as a feedstock could offer cost-
50 competitive production of renewable fuels, chemicals, and materials (Ragauskas et al., 2006).
51 Lignocellulosic biomass is composed mainly of cellulose, hemicellulose, and lignin.
52 Lignocellulosic carbohydrates, cellulose and hemicellulose, can be deconstructed into fermentable
53 sugars for biological conversion into a range of bioproducts. Lignocellulose is a recalcitrant
54 feedstock for biological conversion, in part due to its up to 40% lignin content (Gani and Naruse,
55 2007). Pretreatments are employed prior to carbohydrate deconstruction to increase sugar and thus
56 product yield. However, lignin degradation during pretreatment often produces aromatic
57 compounds that strongly inhibit the growth of most bacteria (Beckham et al., 2016). Moreover,
58 conventional fermentative biorefineries treat lignin as by-product, and burn it for low-value
59 electricity or process heat (Ragauskas et al., 2014). Techno-economic analyses have reported that
60 the use of lignin for co-product generation is a key to profitable lignocellulose valorization
61 (Valdivia et al., 2016). Lignin cannot be rapidly broken down by biological methods (Boerjan et
62 al., 2003; Studer et al., 2011). Thus, an approach for lignin utilization has been proposed that
63 combines the rapid depolymerization kinetics of chemical processing with the metabolic funneling
64 and selective abilities of microbial systems (Linger et al., 2014; Wheeldon et al., 2017). Recently,
65 research groups are attempting to develop microbial hosts with improved tolerance to aromatic
66 compounds for fermentation of lignocellulose-derived sugars and lignin-derived aromatic
67 compounds (Abdelaziz et al., 2016; Ragauskas et al., 2014; Janusz et al. 2017). In particular,
68 lignin-rich streams have been utilized to generate lipid-based biofuels (i.e., waste-to-fuel
69 applications) (Le et al., 2017; Yaguchi et al., 2017).

70 *Rhodococcus opacus* PD630 (hereafter *R. opacus*) is a Gram-positive actinobacterium. It is
71 natively able to utilize sugars, aromatics, furans, and organic acids (Holder et al., 2011; Kurosawa
72 et al., 2015). Isolated from a gas works plant, this strain is capable of accumulating triacylglycerol
73 (TAG), the precursor for biodiesel up to 80% of its dry cell weight (Alvarez et al., 1996). Studies
74 of its mechanisms of aromatic tolerance and TAG accumulation have found promising results for
75 its use in the conversion of lignin-derived aromatic substrates (DeLorenzo et al., 2017; Henson et
76 al., 2018a; Henson et al., 2018b; Yoneda et al., 2016). *R. opacus* has been extensively studied for
77 its growth kinetics and transcriptional activities, yet key knowledge gaps from genotype to
78 phenotype still remain. Specifically, there is little knowledge about the distribution of the
79 metabolic fluxes in this biofuel producer when consuming aromatic substrates. Functional
80 characterization of the central metabolic network is necessary to develop this nonmodel platform
81 for metabolic engineering applications, particularly to facilitate genome-scale modeling for
82 rational strain design.

83 In this work, we have characterized phenol-fed cultures of *R. opacus* based on integrated omics
84 analyses. ¹³C-Metabolic Flux Analysis (¹³C-MFA) can measure *in vivo* reaction rates of central
85 metabolism and confirm global gene regulation patterns inferred from transcriptomic data
86 (Chubukov et al., 2013). In the past, ¹³C-MFA mostly focused on cellular metabolisms fed with
87 sugars, organic acids, and CO₂ (photosynthesis). To our knowledge, this work is the first report of
88 a full MFA completed in any organism using phenol as the sole carbon source. Additionally, we
89 performed ¹³C-pathway tracing, metabolite analysis, flux balance analysis, and biomass
90 composition analysis. The flux-based insights were further connected to recently reported
91 transcriptional profiles and proteomics data. We also examined how adaptive laboratory evolution

92 affects the central flux network. These systems analyses provide a deeper understanding of *R.*
93 *opacus* metabolism of lignin-derived substrates at different layers of cellular processes.

94

95 **Materials and Methods**

96 **Chemicals, strains, and growth conditions.** 1-¹³C phenol, 2,6-¹³C phenol, and U-¹³C phenol were
97 purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). All other chemicals
98 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All cells were grown in minimal
99 media, previously described as media B (DeLorenzo et al., 2017). Unless otherwise noted, the sole
100 carbon source was 0.5 g/L phenol, and the nitrogen source was 1 g/L ammonium sulfate. For all
101 cultures, a single colony of *R. opacus* from a tryptic soy broth plate was inoculated into 2 mL
102 minimal medium, which was used as a seed culture for 10 mL cultures. Isotopomer measurements
103 were conducted when cell cultures reached an optical density at 600 nm (OD₆₀₀) of 0.2-0.5,
104 corresponding to exponential growth.

105

106 **Analysis of proteinogenic amino acid labeling.** 10 mL labeled biomass from 2,6-¹³C phenol or
107 1-¹³C phenol growing cultures (biological duplicates, n=2) were centrifuged down then hydrolyzed
108 at 100 °C for 24 hours. The resulting amino acids were analyzed by GC-MS via the TBDMS (N-
109 (tert-butyldimethylsilyl)-N-methyltrifluoroacetamide) method as described previously (You et al.,
110 2012). In brief, the [m-57]⁺ and [m-159]⁺ fragments for amino acids were mainly used for ¹³C-
111 MFA, while the [m-15]⁺, [m-85]⁺, and f(302)⁺ fragments were also analyzed to provide additional
112 labeling information. The m+0, m+1, m+2, etc. data corresponds to the fraction of fragments that

113 were unlabeled, singly labeled, doubly labeled, etc. Labeling data were corrected for naturally-
114 occurring isotopes.

115

116 **Dynamic labeling (pulse-tracing), free metabolite extraction, and LC-MS analysis.** For phenol
117 dynamic labeling experiments, 20 mL unlabeled phenol cultures were grown to $OD_{600} \sim 0.2$ and
118 then pulsed with 2 mL of a 5.25 g/L $U\text{-}^{13}\text{C}$ phenol stock solution for a final labeled phenol
119 concentration of ~ 0.5 g/L. The cultures were quenched at 5 seconds, 1 minute, and 1 hour using
120 the Fast-Cooling procedure (Hollinshead et al. 2016; Abernathy et al., 2017). To study the effect
121 of benzoate on phenol utilization, 40 mL cultures with 0.5 g/L fully labeled phenol were grown to
122 $OD_{600} \sim 0.35$, and then the labeled cultures were mixed with 4 mL of 4 g/L unlabeled benzoate.
123 After the benzoate pulse, the cultures were quenched at 20 seconds, 1 minute, and 3 hours.

124 At each metabolite quenching time, 10 mL of culture was poured into ice cold carbon-free
125 media B, and then placed into a liquid nitrogen bath. The samples were stirred briefly and then
126 centrifuged (8000 g) at 0 °C for 5 minutes. The pellets were stored at -80 °C until metabolite
127 extraction. To determine labeling in free metabolites, cell pellets were resuspended in 1 mL of 7:3
128 methanol:chloroform, and shaken at 250 rpm at 4 °C for 6 hours with hourly vortexing. The
129 methanol layer was separated by the addition of 500 μL water followed by centrifugation after
130 which the aqueous layer was collected. This separation process was repeated twice. The collected
131 samples were frozen at -80 °C, then lyophilized at -50 °C. The samples were reconstituted with
132 water and analyzed using an Ion Pairing LC-MS method at the Donald Danforth Plant Science
133 Center (Creve Coeur, MO, USA) (Abernathy et al., 2017). To measure metabolite pool size, an
134 isotope ratio approach was used (Bennett et al., 2008; Abernathy et al., 2017). More specifically,
135 *E. coli* was grown on $U\text{-}^{13}\text{C}$ glucose to produce fully-labeled internal standards for each metabolite,

136 while *R. opacus* culture was grown on unlabeled substrates. The cultures were quenched and mixed
137 together at a 1:1 biomass content ratio for metabolite extraction and LC-MS measurements. The
138 relative metabolite pool size was calculated based on ^{13}C ratios.

139

140 **Flux calculations.** Steady-state flux analysis calculations were done using the software INCA
141 (Isotopomer Network Compartmental Analysis) (Young, 2014). The basis of the *R. opacus* flux
142 model is the KEGG genome annotation, including the β -ketoacid pathway, the TCA cycle, the
143 anaplerotic reactions, glycolysis, and the EDP. Carbon mappings for both ortho and meta pathways
144 are shown in **Supplemental Figure 1**. The *R. opacus* central flux network is largely similar to the
145 model bacterial species *E. coli* with major differences in the inclusion of phenol \rightarrow acetyl-CoA +
146 succinyl-CoA, a modified biomass equation (Crown et al., 2015), and the reversibility of glycolysis
147 and the EDP to allow for gluconeogenesis. Labeling data of key proteinogenic amino acids,
148 biomass growth, and substrate consumption rate were used for flux calculation. Based on
149 measurements errors from parallel labeling experiments, 90% confidence intervals of fluxes were
150 calculated via INCA's parameter continuation function (See supplementary Excel file for
151 metabolic reactions, flux constraints, amino acid labeling fitting, and statistical analysis).

152 Flux balance analysis (FBA) of the central metabolic network was done using INCA to
153 further compare actual metabolic fluxes with optimal flux topologies. In addition to maximizing
154 biomass or fatty acid productions, three other objective functions were also tested with fixed
155 biomass growth rate: maximizing NAD(P)H, ATP, or minimizing sum of fluxes (i.e., enzyme
156 usage). The FBA results were compared to ^{13}C -MFA by the sum of squared residuals between
157 optimal flux and measured flux in central pathways.

158

159 **Biomass composition analyses and substrate/product concentration measurements.** Cell
160 cultures were grown to exponential phase, pelleted, washed with sterile water and then lyophilized.
161 Amino acid composition analysis was performed by the Molecular Structure Facility, University
162 of California (Davis, CA, USA). Fatty acid composition analysis was performed by Microbial ID
163 (Newark, DE, USA). Overflow metabolites were analyzed using enzyme kits from R-Biopharm
164 (Germany). Phenol and benzoate in the culture supernatant were derivatized using methyl
165 chloroformate (Madsen et al., 2016) and quantified using a custom GC-MS-FID system (gas
166 chromatography-mass spectrometry-flame ionization detector). Briefly, the culture supernatant
167 was centrifuged at 16,000 g for 5 min, and 200 μ L of the culture supernatant was mixed with 40
168 μ L of 5.0% (w/w) sodium hydroxide solution, 200 μ L of methanol, and 50 μ L of pyridine. Methyl
169 chloroformate (50 μ L) was added to the mixture in two 25 μ L aliquots. Next, 400 μ L of chloroform
170 containing a decane internal standard was added to the sample, followed by the addition of 400 μ L
171 of 50 mM sodium bicarbonate solution to induce phase separation of the aqueous and organic
172 layers. Samples were vortexed between each step to ensure complete mixing. After phase
173 separation, the organic phase was transferred to a GC vial with a 350 μ L glass insert (Agilent), and
174 samples were analyzed using an Agilent 7890A GC coupled to both an Agilent 5975C mass
175 spectrometer containing a triple-axis detector and an Agilent G3461A FID detector with a
176 methanizer (Activated Research Company; Eden Prairie, MN, USA). The Agilent 7890A GC was
177 equipped with a Restek fused silica RTX-50 capillary column (30 m by 0.25 mm, 0.5 μ m film
178 thickness), and helium was used as the carrier gas. 1 μ L of the organic phase was injected with a
179 splitting ratio of 10:1 using the autosampler. For GC runs, the inlet was maintained at 250 $^{\circ}$ C, and
180 the oven was held for 2 min at 40 $^{\circ}$ C, heated to 300 $^{\circ}$ C using a 5 $^{\circ}$ C/min ramp, and held at 300 $^{\circ}$ C

181 for 5 min. All data was exported and analyzed using the Agilent ChemStation Software, and peak
182 intensities were normalized to the decane internal standard. Phenol and benzoate were identified
183 based on retention time and concentrations were determined using external standard curves.

184

185 **RNA-seq analysis.** Raw transcriptomic sequences were downloaded from the Sequence Read
186 Archive (SRA) accession number SRP131196 (Henson et al., 2018a). Sequences were quality
187 trimmed using Trimmomatic (Bolger et al., 2014) before mapping to the Chinese Academy of
188 Science reference genome of *R. opacus* (ASM59954v1) (Chen et al., 2014) using Bowtie2
189 (Langmead and Salzberg, 2012). The resulting SAM files were converted to the BAM format and
190 indexed using SAMtools (Li et al., 2009). Expression counts were then calculated using
191 featureCounts (Liao et al., 2014) and normalized using DESeq2 (Love et al., 2014). **Supplemental**
192 **Table 1** summarizes RNA-seq data of *R. opacus* strains growing with glucose and phenol.

193

194 **Results and Discussion**

195 **Cell growth and biomass composition.** The glucose metabolism of *R. opacus* has been studied
196 previously (Hollinshead et al., 2015). Here, we investigated the growth rates of *R. opacus* on
197 acetate, phenol, phenol with glucose, and glucose (**Table 1**). The fastest growth rate (0.24 hr^{-1}) for
198 wild type *R. opacus* was observed when glucose was provided as the sole carbon source. Growth
199 on phenol was, as expected, slower (0.17 hr^{-1} , $P=0.013$; two-tailed Student's *t* test), likely owing
200 to a combination of its toxic effects and less efficient phenol degradation processes. When phenol
201 is provided in combination with glucose, these disadvantages are largely mitigated – the rapidity
202 with which glucose can be processed apparently compensated for the kinetic disadvantages of

203 phenol metabolism (e.g., requirement of gluconeogenesis), which allowed the cultures to grow
 204 nearly as quickly as the glucose-only case ($P=0.46$). Moreover, the co-utilization of both substrates
 205 produced more biomass at the end of the cultivations ($P<0.01$). Interestingly, acetate-fed cultures
 206 showed similar growth performance to phenol-fed cultures ($P=0.65$). The TCA cycle is the
 207 entering point of the central flux network for both acetate and phenol, but phenol utilization
 208 requires more conversion steps than acetate consumption. Therefore, the similar growth rates
 209 indicate that the β -ketoacid pathway in *R. opacus* is as efficient as the acetate conversion route
 210 (acetate \rightarrow acetyl-CoA).

211

212 **Table 1. A summary of the growth rate, corresponding doubling times (t_D), and maximum**
 213 **optical density of *R. opacus* on four different carbon substrates.** All data presented is the
 214 average of biological triplicates \pm standard deviation.

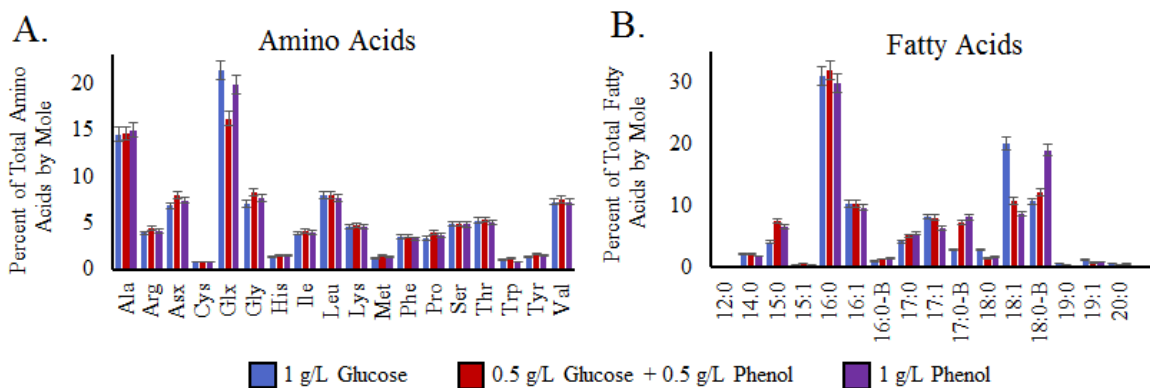
Carbon Substrate	Growth Rate (hr^{-1})	t_D (hr)	Maximum OD ₆₀₀
2.0 g/L Acetate	0.16 ± 0.02	4.30	1.08 ± 0.001
0.5 g/L Phenol	0.17 ± 0.02	4.10	0.82 ± 0.02
0.3 g/L Phenol + 1.0 g/L Glucose	0.24 ± 0.01	2.96	1.53 ± 0.01
1.0 g/L Glucose	0.25 ± 0.02	2.81	1.20 ± 0.02

215

216 A biomass composition analysis was conducted on cultures grown in phenol, glucose, and a
 217 phenol-glucose mixture. The data from the phenol culture was used to modify the biomass equation
 218 in the metabolic flux model. **Figure 1A** shows the proteinogenic amino acid composition of *R.*
 219 *opacus* from three tested samples, where glutamate/glutamine and alanine were most abundant.
 220 Proteinogenic amino acid synthesis in *R. opacus* undergoes minimal changes when grown on
 221 different carbon sources. *R. opacus* is known to store high concentrations of lipids in late growth

222 phases, and reportedly can accumulate lipids up to 80% of its dry cell weight under low-nitrogen
223 stress (Alvarez et al., 1996; Henson et al., 2018a). In the exponential growth phase when nitrogen
224 is still available, ~0.3 g lipid per gram dry cell weight was accumulated in biomass samples. We
225 further probed the fatty acid profiles of *R. opacus* when fed the three previously-described carbon
226 sources with sufficient nitrogen (**Figure 1B**). In general, the most abundant fatty acid (in the form
227 of acyl groups of lipids) is palmitic acid (16:0), accounting for approximately 30% of total lipid
228 fatty acids. Compared to the model bacterium *E. coli*, *R. opacus* contains higher proportions of
229 long-chain fatty acids (**Supplemental Figure 2**). Specifically, 47% of fatty acids in *R. opacus*
230 were found to contain 17 carbons or more, whereas only 12% of fatty acids in *E. coli* have over 16
231 carbons (Neidhardt et al., 1990). Additionally, *R. opacus* natively produces C15 fatty acids and
232 branched-chain fatty acids (BCFAs) containing 16, 17, and 18 carbon atoms. Unlike most Gram-
233 positive bacteria and some engineered Gram-negative strains which only produce terminal BCFAs
234 (Bentley et al., 2016; Jiang et al., 2015; Kaneda, 1966), *R. opacus*' BCFAs have internal branches,
235 which are expected to produce biofuels with better cold-flow properties than terminally-branched
236 fuels (Pond and Langworthy, 1987). The carbon source affected the abundance of some fatty acids.
237 Notably, ~20% of the total fatty acids in phenol-fed culture were C18 BCFAs.

238



239

240 **Figure 1. The distribution of amino acids (A) and fatty acids (B) in *R. opacus* with three**
 241 **different carbon sources.** Asx = aspartate/asparagine and Glx = glutamate/glutamine; fatty acids
 242 are denoted as carbon chain length: number of unsaturated bonds, with a B indicating branching.
 243 Error bars are estimates of technical uncertainty.

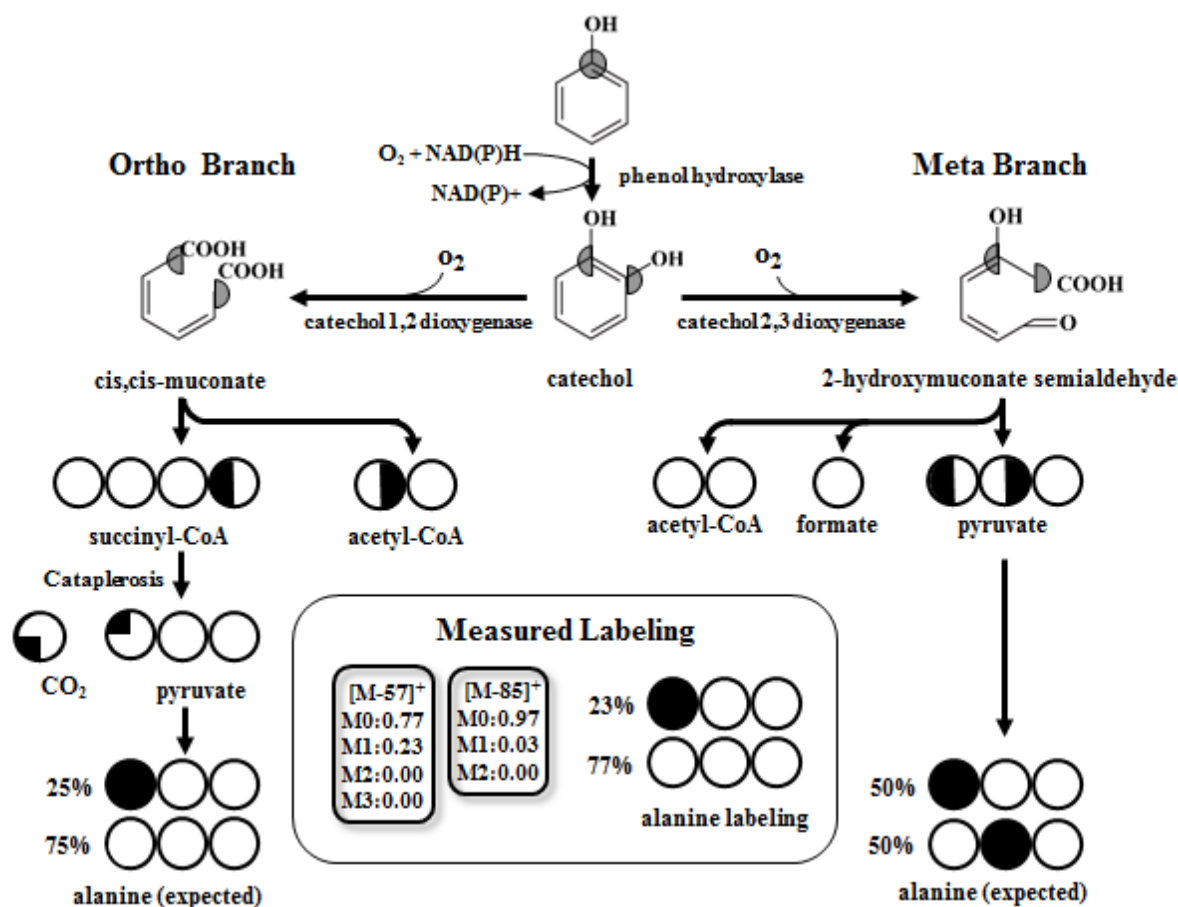
244

245 **Phenol degradation via the β -keto adipate pathway.** The enzymatic pathways to degrade
 246 aromatic substrates can be grouped into categories based on the position of bond cleavage (Fuchs
 247 et al., 2011). The KEGG annotation for *R. opacus* contains every gene for the meta cleavage of
 248 catechol, while one of the genes in the ortho pathway, 3-oxoadipate CoA-transferase, is
 249 unannotated. Both pathways begin with a two-component flavoprotein monooxygenase, phenol
 250 hydroxylase, which oxidizes the phenol ring to form catechol (**Figure 2**). Saa et al. studied this
 251 enzyme in *Rhodococcus erythropolis* and found that it can accept electrons from both NADH and
 252 NADPH to cleave O₂ molecules. However, phenol hydroxylase's affinity for NADH was
 253 measured to be 5-10 times greater than its affinity for NADPH (Saa et al., 2010). This may be an
 254 evolutionary adaptation to avoid competition for NADPH between phenol consumption and
 255 biomass formation. The ortho and meta pathways diverge with the ortho branch employing
 256 catechol-1,2-dioxygenase and the meta branch using catechol-2,3-dioxygenase. The intermediates
 257 in the ortho pathway include cis-cis muconic acid and β -keto adipate, and its end products are
 258 acetyl-CoA and succinyl-CoA. In contrast, the meta pathway's intermediates include 2-

259 hydroxymuconic semialdehyde, and its final products are pyruvate, formate, and acetyl-CoA
260 (Sridevi et al., 2012). Meta-cleavage has been found in *Pseudomonas* species to degrade phenol
261 and phenol derivatives (Kukor and Olsen, 1991). To determine which route is used by *R. opacus*,
262 we used 1-¹³C phenol labeling (**Figure 2**). The symmetry of catechol results in two different
263 alanine labeling patterns for each branch. In the ortho pathway, 50% of labeled carbon will end up
264 as the 4th position carbon of succinyl-CoA and the remaining 50% will occupy the 1st position of
265 acetyl-CoA. In the TCA cycle, succinyl-CoA is converted to succinate, another symmetric
266 molecule. The reactions of cataplerosis, e.g., the phosphoenolpyruvate (PEP) carboxykinase-
267 catalyzed reaction, release the 4th position carbon as CO₂, and convert the remaining 3-carbon
268 fragment to pyruvate. The expected labeling pattern for pyruvate derived from the ortho branch is
269 therefore 25% first-position labeled and 75% unlabeled. In contrast, the meta-cleavage will create
270 pyruvate molecules with an even split between those labeled in the first and the second positions.
271 Alanine's sole precursor is pyruvate, and thus pyruvate's labeling can be determined by measuring
272 alanine. Our analysis showed that ~23% of alanine was labeled only at the 1st position, and ~77%
273 of alanine was unlabeled. This provides strong evidence that the ortho pathway is the exclusive
274 route for phenol degradation. The labeling result is consistent with our previous gene knockout
275 analyses (Henson et al., 2018a). Specifically, when muconate cycloisomerase, an ortho pathway
276 enzyme, was disrupted, *R. opacus* was no longer able to grow on phenol. Additionally, proteomic
277 data from this species has shown that several enzymes in the ortho pathway were abundant during
278 aromatic metabolism (Xie et al., 2019). The missing ortho pathway reaction (3-oxoadipate → 3-
279 oxoadipyl CoA) could be catalyzed by succinyl-CoA:3-ketoacid-CoA transferase
280 (PD630_RS25340 + PD630_RS25345) (Yoneda et al., 2016). We also found that the presence of
281 low concentration formate (0.5 g/L) was inhibitory to cell growth, implying that the meta pathway

282 with byproduct formate is unlikely to be beneficial for growth. It is worth noting that the genes
 283 coding the ortho pathway enzymes are found in two distinct clusters on the chromosome, while
 284 the genes for the meta pathway are scattered across the genome and two endogenous plasmids. All
 285 these analyses prove the value of ^{13}C tracing to delineate functional pathways and to fill the
 286 annotation gaps.

287



288

289 **Figure 2. ^{13}C phenol catabolism in *R. opacus* is through the ortho-cleavage.** GC-MS data of
 290 alanine gives labeling data for pyruvate. The $[\text{M}-57]^+$ and $[\text{M}-85]^+$ fragments were used to
 291 determine the labeling of each carbon of pyruvate. The labeling of pyruvate closely matches the
 292 expected labeling for phenol catabolism through the ortho branch. A detailed tracing is shown in
 293 **Supplemental Figure 1.**

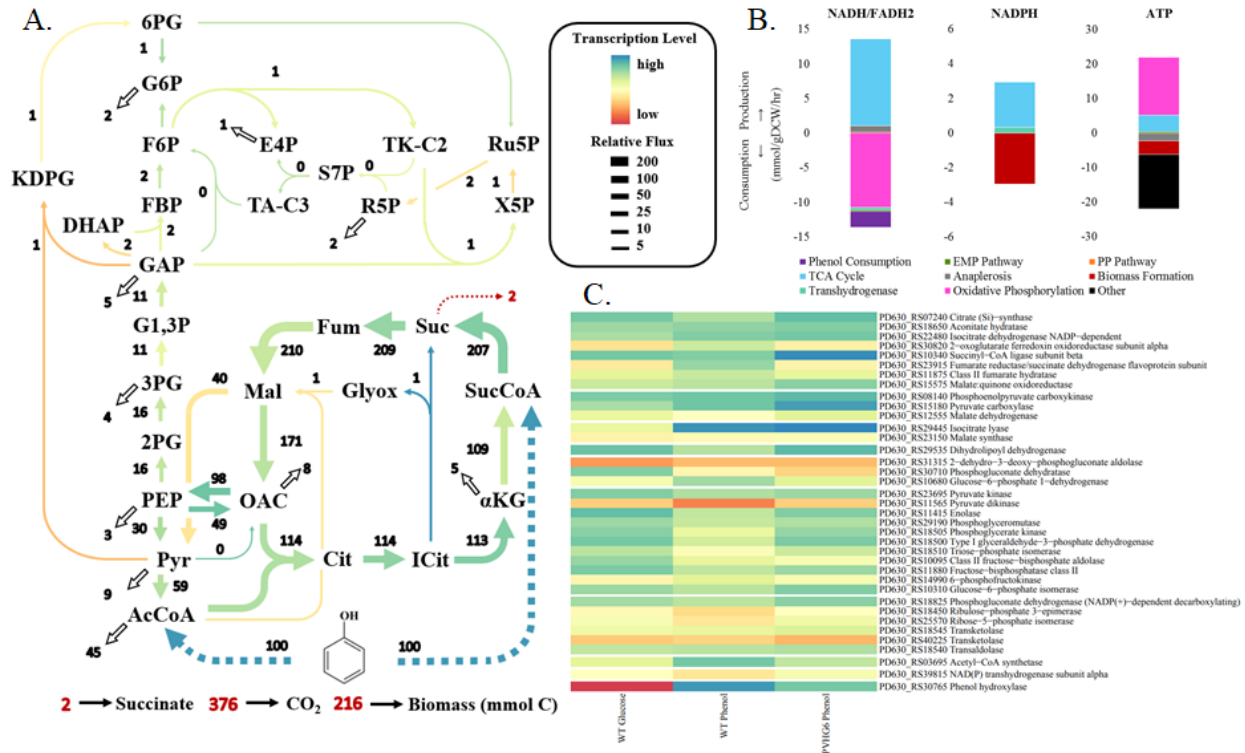
294

295 ***R. opacus* flux map.** ^{13}C -MFA was performed to comprehensively investigate *R. opacus*' phenol
296 metabolism (**Figure 3A**). The metabolic model had 71 reactions and 33 independent parameters,
297 and was constrained by 174 independent mass isotopomer measurements, resulting in 141 degrees
298 of freedom. Amino acid labeling data with 0.75 mol % measurement error from parallel tracer
299 experiments ($1\text{-}^{13}\text{C}$ Phenol and $2,6\text{-}^{13}\text{C}$ Phenol) was used to generate the flux map. The SSR (sum
300 of square residuals) for the presented fit was 119.4, which is inside the 90% confidence bounds for
301 the SSR [114.6 169.7]. Experimentally, the rate of phenol uptake (i.e., the amount of phenol
302 entering the central metabolism per cell mass within a given time) at exponential phase was
303 measured to be 2.3 mmol/hr/g dry cell weight (**Supplemental Figure 3**). ^{13}C -MFA shows that per
304 100 mmol of phenol consumed ~2 mmol extracellular overflow metabolites (i.e., succinate), ~370
305 mmol CO_2 , and ~220 mmol C of biomass (~4.7 g) are produced, resulting in a ~50% yield of
306 biomass from phenol (**Figure 3A**).

307 The TCA cycle of *R. opacus* in phenol culture has very high fluxes to sustain aerobic
308 growth. The citrate entrance node has a relative flux over 100, which is much higher than reported
309 fluxes in glucose-fed *E. coli* (Crown et al., 2015). The direct infusion of succinyl-CoA from phenol
310 degradation drives high fluxes through succinyl-CoA ligase, succinate dehydrogenase, and
311 fumarate hydratase in the TCA cycle and causes succinate overflow (**Supplemental Figure 4**). In
312 the supernatant, α -ketoglutarate could also be detected while acetate secretion was not observed.
313 The overflow of the TCA cycle works to balance excessive succinyl-CoA flux from phenol
314 degradation. In contrast, excessive acetyl-CoA (i.e, not forming citrate in the TCA cycle) enters
315 into biomass pools (lipid and amino acids). Further, the glyoxylate shunt is measurable, offering
316 an alternative route for acetyl-CoA consumption. On the other hand, several anaplerotic reactions
317 are also active to direct flux outside the TCA cycle (note: the confidence intervals of these fluxes

318 are relatively wide due to limited labeling resolution). For instance, the malic enzyme is active to
319 pump the TCA fluxes for pyruvate synthesis. Moreover, both ^{13}C -MFA and gene expression data
320 identified the GTP-dependent PEP carboxykinase (PD630_RS08140) as the driving force for
321 gluconeogenesis (OAC \rightarrow PEP) and flux towards the upper pathways (the EDP and the pentose
322 phosphate pathways). Interestingly, the model shows that the EDP, operating in reverse, can serve
323 as a secondary route to the upper pathways. Fluxes in the upper pathways of phenol-fed cultures
324 are small and primarily used for biomass synthesis.

325 To offer a deeper understanding of *R. opacus* flux network optimality, the ^{13}C -MFA model
326 was compared to flux balance analysis using five different objective functions (**Supplemental**
327 **Figure 5**) (Schuetz et al., 2007). Based on the SSRs between the MFA fluxes and FBA fluxes, the
328 metabolism of *R. opacus* is most closely optimized to maximize the production of energy
329 molecules rather than the production of biomass or fatty acids or the minimization of enzyme use
330 (**Supplemental Figure 6**). The high TCA cycle fluxes elucidated by ^{13}C -MFA facilitate NADH,
331 and subsequently ATP production. The elevated priority of energy molecules is the tradeoff against
332 biomass growth with stress inducing aromatic substrates. Moreover, *R. opacus* flux topology does
333 not gear towards minimal usage of enzymes, allowing for metabolic flexibility to utilize
334 unconventional substrates.



335

336 **Figure 3. Flux and transcriptional analyses of *R. opacus* strains.** (A) *R. opacus* PD630's flux
 337 map when phenol is the sole carbon source. The flux values are relative flux distributions based
 338 on 100 mmol of phenol consumed by the cell to generate 100 mmol of influx toward both acetyl-
 339 CoA and succinyl-CoA. Arrow color corresponds to the relative gene expression levels of the
 340 enzyme that catalyzes the reaction (yellow/orange=low and green/blue=high). Arrow size
 341 corresponds to relative flux. White arrows are fluxes to biomass formation. The dashed red arrow
 342 is flux out of the cell. (B) The major sources and sinks of NADH, NADPH, and ATP in *R.*
 343 *opacus*'s phenol metabolism (note: pyruvate dehydrogenase reaction was lumped with the EMP
 344 pathway). (C) The transcript levels from wild type and an adapted strain (PVHG6) *R. opacus*
 345 grown on glucose or phenol were compared for the following metabolisms: TCA cycle, anaplerotic
 346 pathways, the glyoxylate shunt, the EMP, the EMP pathway, the pentose phosphate pathway,
 347 transhydrogenation, and phenol consumption (Henson et al., 2018a).

348

349

350 **Cofactor balance and energy metabolism.** Phenol metabolism carries very low fluxes through
 351 the oxidative pentose phosphate pathway, which is the major NADPH source for common glucose-
 352 utilizing microbes (such as *E. coli*). Instead, significant TCA fluxes are responsible for generating
 353 large amounts of NADH, NADPH, and ATP (**Figure 3B**). Unlike NADPH, which is primarily

354 consumed in the formation of biomass, NADH is used for the oxidation of phenol to catechol and
355 the production of ATP. *R. opacus* can use NAD-dependent malic enzyme (PD630_RS12555, EC
356 1.1.1.38), but lacks NADP-dependent malic enzyme (EC 1.1.1.40). Consequently, most NADPH
357 generation occurs in the TCA cycle by NADPH-dependent isocitrate dehydrogenase
358 (PD630_RS13780), which explains the necessity to maintain high flux through the TCA cycle. A
359 search of *R. opacus*'s genome identified NAD(P) transhydrogenase subunit alpha
360 (PD630_RS39815). The transhydrogenase might further fine-tune the cofactor balance. On the
361 other hand, ATP can be generated directly by the TCA cycle (as GTP) and indirectly through
362 oxidative phosphorylation. ATP is needed mainly for biomass formation (in particular for lipid
363 synthesis) and other cellular processes (e.g., cell maintenance, substrate active transport, and
364 gluconeogenesis). Additionally, we calculated the intracellular energy charge based on LC-MS
365 measurements to determine the relative concentrations of ATP, ADP, and AMP (*Energy Charge*
366 $= \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$) (Supplemental Figure 7). Phenol-fed cultures have an energy
367 charge of ~0.9, indicating that the concentration of ATP is much greater than that of ADP and
368 AMP combined. Glucose-fed cultures have a comparable energy charge (~0.85). An explanation
369 for this similarity is that, during aerobic growth, the NADH synthesized from either substrate can
370 be effectively used via oxidative phosphorylation to generate ATP (Figure 3B). These results
371 along with FBA results demonstrate that *R. opacus* has sufficient energy carrying molecules to
372 support the production of high energy biofuels from different feedstocks.

373
374 **RNA-seq analysis of flux network.** RNA-seq was used to compare transcriptional regulation
375 between conditions (Figure 3, Supplemental Table 1). It is important to note that while

376 transcriptomics can illuminate how genes are regulated, it does not strictly correlate to enzyme
377 activities or flux values due to variations in translational rate and allosteric regulation. Using data
378 generated in the previous report, we compared the transcription levels of central pathway enzymes
379 in glucose- and phenol-fed cultures of wild type *R. opacus* and *R. opacus* PVHG6, a mutant strain
380 which was adaptively evolved for improved growth on a mixture of aromatic compounds (**Figure**
381 **3C**) (Henson et al., 2018a). When consuming glucose, *R. opacus* expressed phenol hydroxylase at
382 low levels, while its key enzymes for glycolysis are mostly highly expressed. During phenol
383 utilization, *R. opacus*' isocitrate lyase (PD630_RS29445) was highly expressed, but the glyoxylate
384 shunt flux was low.

385 Gluconeogenesis, which is required when phenol is the sole carbon source, is typically thought to
386 occur through the EMP pathway operating in reverse. However, the ¹³C-MFA model suggests that
387 the EDP enzymes (in the reverse direction) also play a role in *R. opacus*. Transcriptomic data was
388 used to investigate this finding. Despite its low transcription levels in glucose cultures, the EDP
389 was found to be the main route for glucose consumption in *R. opacus* (Hollinshead et al., 2015).
390 The EDP has comparable transcription levels in phenol-fed cultures and glucose cultures, and this
391 suggests that it is active in the reverse direction when phenol is the carbon source. When running
392 in reverse, the EMP pathway uses most of the same enzymes as it does when it runs forward, with
393 the exception of conversion between FBP to F6P and pyruvate to phosphoenolpyruvate (PEP). In
394 the glycolytic direction, F6P is converted to FBP by 6-phosphofructokinase (PD630_RS14990);
395 in the gluconeogenic direction, FBP → F6P occurs via fructose-1,6-bisphosphatase
396 (PD630_RS11880). Interestingly, the number of transcripts of fructose-1,6-bisphosphatase is
397 lower in phenol cultures than in glucose cultures (**Supplemental Table 1**), implying fructose-1,6-
398 bisphosphatase activity is reduced during phenol metabolism. Additionally, the gene encoding

399 pyruvate dikinase (PD630_RS11565, pyruvate \rightarrow PEP) has very low expression levels during
400 phenol metabolism. Therefore, the anaplerotic reaction via phosphoenolpyruvate carboxykinase
401 (PD630_RS08140, oxaloacetate \rightarrow PEP) is indispensable for supporting gluconeogenic fluxes.
402 Intriguingly, the aromatic mixture adapted strain, PVHG6, was found to express pyruvate dikinase
403 and succinyl-CoA ligase at higher rates than the wild type in phenol culture, which may facilitate
404 aromatic utilization and tolerance.

405

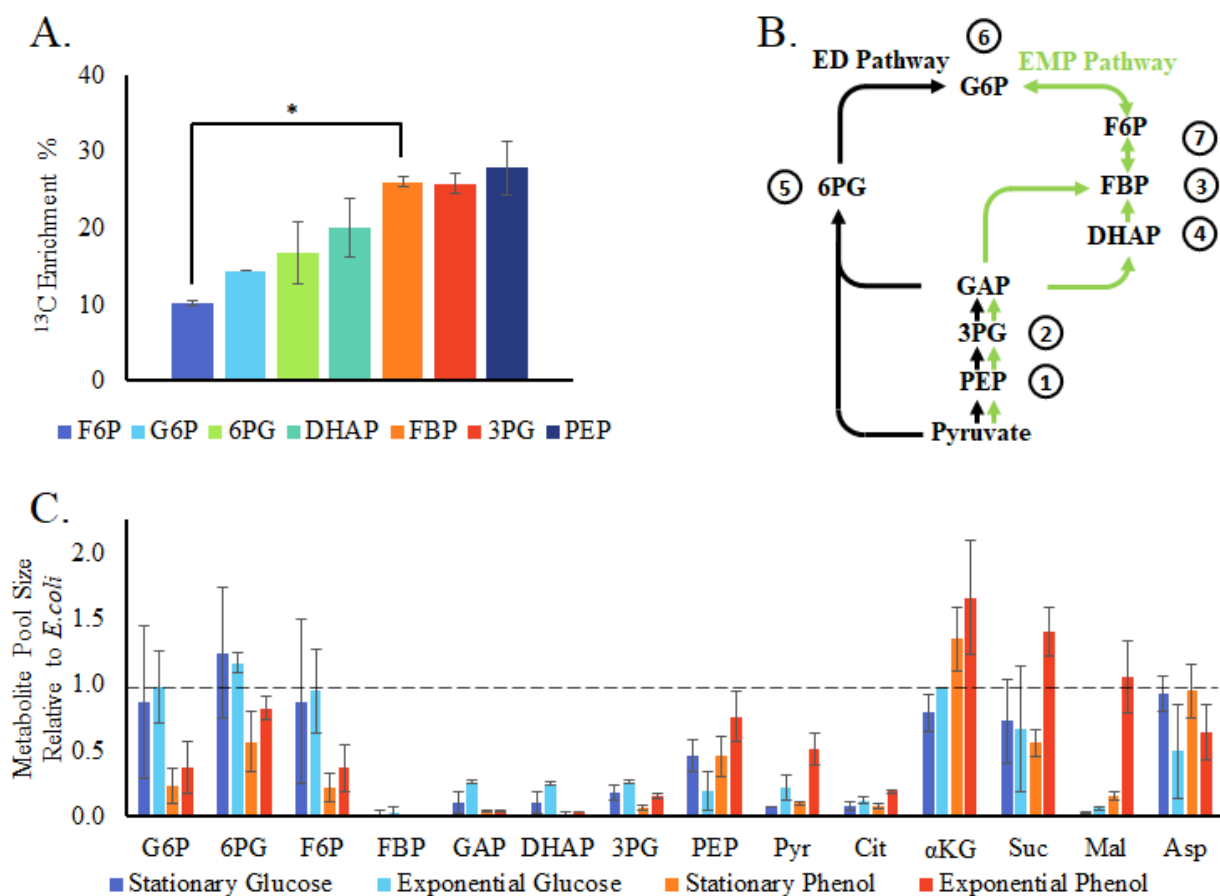
406 **Pulse-Trace labeling and metabolite pool measurement.** The gluconeogenesis pathways were
407 further investigated with a ^{13}C -pulse experiment (**Figure 4A, 4B, Supplemental Figure 8**).
408 Conceptually, metabolites with the highest flux are expected to incorporate ^{13}C carbon most
409 rapidly. The labeling order of glycolysis metabolites observed in phenol-fed cultures is the reverse
410 of that in glucose-fed metabolism: phosphoenolpyruvate (PEP) was labeled first followed closely
411 by 3-phosphoglycerate (3PG). Fructose-bis-phosphate (FBP) was found to be labeled significantly
412 faster than fructose-6-phosphate (F6P) ($P=0.03$). This may be due to a combination of the
413 thermodynamic barrier between FBP and F6P (i.e., reaction Gibbs free energy favors flux from
414 F6P to FBP), the small pool size of FBP (**Figure 4C**), and the low transcription levels of fructose-
415 1,6-bisphosphatase (**Figure 3C, Supplemental Table 1**).

416 The intracellular metabolite concentrations, or pool sizes, were measured for *R. opacus*
417 cultures grown on glucose and phenol at stationary and exponential time points (**Figure 4C**). Cells
418 growing exponentially on phenol have very high quantities of TCA cycle intermediates. However,
419 their intracellular concentrations of fructose-bis-phosphate (FBP), dihydroxyacetone phosphate
420 (DHAP), and glyceraldehyde-3-phosphate (GAP) are at minimum 10-fold lower than those of *E.*
421 *coli*. This offers thermodynamic evidence for high TCA cycle fluxes paired with low reverse EDP

422 and gluconeogenic fluxes. Compared to glucose-fed *E.coli*, phenol-fed *R. opacus* has relatively
 423 low citrate concentrations. This may be the result of a highly active isocitrate dehydrogenase, an
 424 enzyme needed for both α KG and NADPH production. Also, *R. opacus* has relatively high
 425 concentrations of α -ketoglutarate (α KG), especially during exponential growth. An explanation is
 426 that succinyl-CoA/succinate accumulation from phenol degradation inhibits α -ketoglutarate
 427 dehydrogenase (α KG \rightarrow succinyl-CoA), leading to excess amounts of α KG (note: both succinate
 428 and α KG were detected in the supernatant). Therefore, succinyl-CoA is likely a bottleneck node
 429 causing metabolite overflow during phenol metabolism.

430

431



432

433 **Figure 4. Dynamic labeling and metabolite pool size measurement.** (A) Pulse-trace labeling
434 data from cultures that were quenched within one minute after ^{13}C phenol was added. Asterisk (*)
435 indicates that the difference between F6P and FBP values is statistically significant ($P < 0.05$, one
436 mean, two-tailed Student's t-test). (B) A simplified metabolic map of the ED and EMP pathways
437 annotated with 1 being the fastest labeled metabolite and 7 being the slowest. (C) Intracellular
438 metabolite concentrations relative to those in glucose-fed *E. coli* (used as internal standards).

439

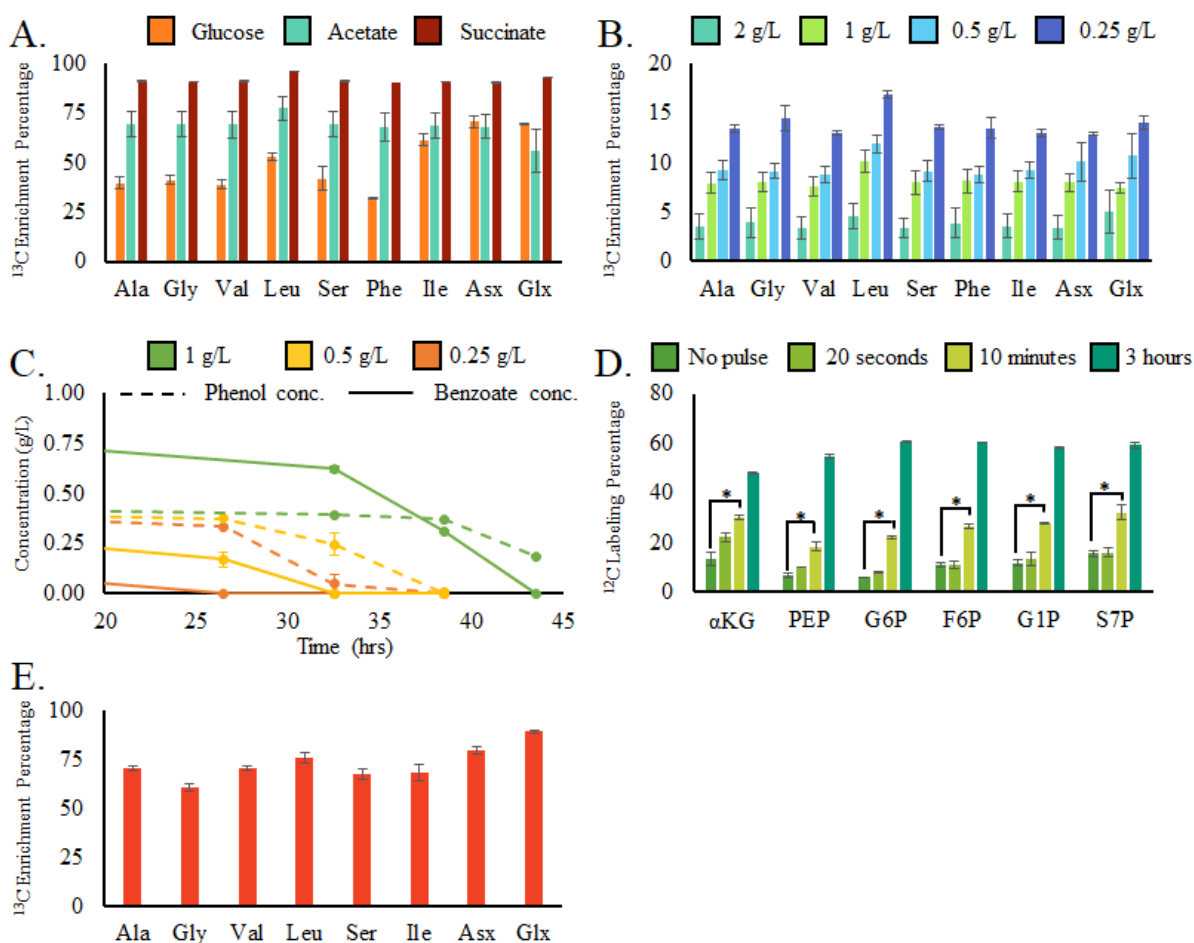
440 **Phenol co-utilization with other substrates.** Industrial applications of *R. opacus* will require
441 growth on lignin hydrolysates, which contain a heterogeneous mixture of compounds (Beckham
442 et al., 2016). Notably, *R. opacus* can consume aromatic compounds simultaneously with glucose
443 without exhibiting catabolite repression (Hollinshead et al., 2015). However, it was found that *R.*
444 *opacus* consumes some aromatic compounds sequentially, rather than concurrently (Henson et al.,
445 2018a). Here we further investigated co-metabolism of phenol with competing substrates (glucose,
446 acetate, succinate, and benzoate) (**Figure 5A, 5B, 5C**). Our findings for phenol-glucose cultures
447 are consistent with the previous report, finding no interaction between the two substrates. A
448 separate study observed that phenol degradation enzyme transcripts in *Rhodococcus erythropolis*
449 CCM2595 were downregulated in the presence of succinate (Szököl et al., 2014). Yet, when
450 phenol and succinate were co-fed in our experiment, phenol was dominantly used (>90% of carbon
451 originated from phenol). Since phenol metabolism produces excessive succinate, *R. opacus* may
452 repress the utilization of external succinate in order to preferentially consume the toxic compound.
453 Similarly, *R. opacus* can co-utilize phenol and acetate, and phenol is preferred (~70% of
454 proteinogenic carbon originated from phenol). On the other hand, benzoate was found to be
455 utilized faster than phenol (**Figure 5B, 5C**).

456 Three experiments were performed to elucidate the interaction of phenol and benzoate. In
457 the first experiment, amino acid labeling was used to quantify the relative uptake rate between
458 benzoate and phenol (**Figure 5B**). We found that the ratio of benzoate/phenol uptake rate

459 (calculated via ^{13}C labeling data) during early growth phase showed a linear relation to the
460 concentration ratio of the two substrates in the media (**Supplemental Figure 9**). When cells grew
461 in media containing equal moles of fully labeled phenol and unlabeled benzoate, the proteinogenic
462 amino acids contained ~90% ^{12}C carbon from benzoate. This result indicates that the wild type
463 strain consumes both substrates, but its benzoate utilization is much faster than its phenol
464 utilization. The second experiment examined how the concentrations of benzoate and phenol in
465 the media decreased over time (**Figure 5C**). We validated that benzoate serves as the main carbon
466 source when both benzoate and phenol are present: phenol was consumed slowly in the presence
467 of benzoate, and its consumption increased once benzoate was nearly exhausted. Lastly, a benzoate
468 pulse was given into fully-labeled phenol-fed, exponentially growing cultures to investigate the
469 rate at which unlabeled benzoate enters into the central metabolism of *R. opacus* (**Figure 5D**). To
470 consider contribution of CO_2 fixation to the unlabeled carbon percentage (Hollinshead et al.,
471 2015), we included no-pulse controls with which the benzoate-pulsed cultures were compared
472 (using one mean, two-tailed Student's t-test). When the phenol growing *R. opacus* was quenched
473 within 20 seconds of a benzoate pulse, little amount of carbon from benzoate had entered the
474 central metabolism (e.g., P values = 0.11 for αKG). In contrast, at 10 minutes, a significant amount
475 of carbon had been incorporated into central metabolites from the added benzoate ($P < 0.05$ for
476 αKG , PEP, and G6P), which indicate phenol catabolism in *R. opacus* could be quickly shifted to
477 benzoate utilization without long lag phase.

478 The downstream products of phenol are acetyl-CoA and succinyl-CoA. To investigate how
479 these two substrates interact, we grew *R. opacus* in media containing fully labeled acetate and
480 unlabeled succinate (1 g/L each) (**Figure 5E**). We hypothesized that acetate would be preferred
481 over succinate since succinate is not fully consumed in phenol metabolism. GC-MS measurements

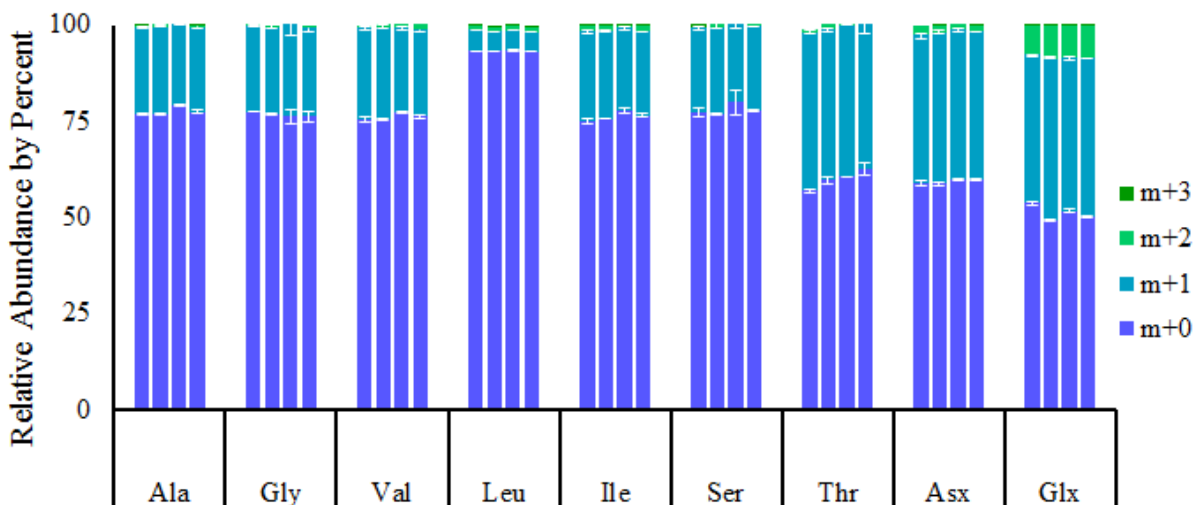
482 showed that *R. opacus* prefers acetate, as the carbon in its amino acids was mostly derived from
 483 acetate (~70%). This observation further confirmed that the metabolite nodes succinate/succinyl-
 484 CoA might be the rate limiting steps during phenol metabolism, leading to flux congestion.
 485
 486



487
 488 **Figure 5. Mixotrophic phenol metabolism.** (A) The contribution of phenol-derived carbon to
 489 amino acids in the presence of 0.5 g/L U-¹³C phenol and 1 g/L of unlabeled glucose, acetate, or
 490 succinate. Samples were taken at early exponential growth (OD₆₀₀=0.4~0.5) when both substrates
 491 remained in the media. (B) The contribution of phenol to the production of amino acids in the
 492 presence of 0.5 g/L U-¹³C phenol and decreasing concentrations of unlabeled benzoate. Samples
 493 were taken at early exponential growth phase (OD₆₀₀=0.25~0.3) when both substrates remained in
 494 the media. The bars correspond to the relative uptake rate of phenol compared to that of benzoate.

495 (C) The supernatant concentrations of phenol and benzoate over time when the starting
 496 concentration of U-¹³C phenol was 0.5 g/L and the starting concentration of benzoate was varied.
 497 (D) The contribution of benzoate-derived carbon to central metabolites at 20 seconds, 10 minutes,
 498 and 3 hours after a benzoate pulse into phenol-fed cultures. Asterisk (*) indicates that the
 499 difference between labeling is statistically significant (P < 0.05, one mean, two-tailed Student's t-
 500 test) (E) The contribution of acetate to amino acids in the presence of 1 g/L U-¹³C acetate and 1
 501 g/L unlabeled succinate. Figures 5A, 5B, 5C, and 5D represent data from biological duplicates.
 502 Figure 5E represents data from technical triplicates.

503



504

505 **Figure 6. The amino acid labeling pattern of wild type *R. opacus* and three mutant strains**
 506 **when grown on 1-¹³C phenol** (From left to right: wild type, phenol-adapted strain P1, benzoate-
 507 adapted strain B2, and aromatic-mixture-adapted strain PVHG6; m+n = unlabeled mass m plus n
 508 labeled carbons).

509

510 **Mutant flux network after adaptive evolution.** In a previous report, *R. opacus* was adaptively
 511 evolved to tolerate high concentrations of various aromatic compounds, including phenol,
 512 benzoate, vanillate, 4-hydroxybenzoate, and guaiacol (Henson et al., 2018a). Three mutant strains
 513 (P1, B2, and PVHG6) were obtained via adaptive evolution on increasing concentrations of phenol
 514 (P), benzoate (B), or a mixture of phenol, vanillate, 4-hydroxybenzoate, and guaiacol (PVHG),
 515 respectively. These mutant strains were found to have distinct mutations and different growth

516 profiles when provided with a mixture of phenol, vanillate, 4-hydroxybenzoate, guaiacol, and
517 benzoate as a carbon source. To determine whether these mutations altered flux through central
518 pathways, these evolved strains were grown on 0.5 g/L of 1-¹³C phenol, and the resulting amino
519 acid labeling patterns were compared to that of the wild type strain (**Figure 6**). Adapted stains, P1
520 and PVHG6 showed similar growth rates on phenol as that of the wild type ($P=0.70$ and $P=0.11$,
521 respectively) (**Supplemental Table 2**). The benzoate-adapted strain showed reduced growth on
522 phenol compared to the wild type strain, only reaching an OD₆₀₀ value of 0.2 after 50 hours
523 ($P=3.7\times 10^{-5}$), and a maximum growth rate of 0.10 hr⁻¹ ($P=0.01$). This supports previous findings
524 that while adaptive evolution can help improve specific microbial traits, its untargeted nature can
525 result in unexpected negative consequences or tradeoffs in other traits (Yi and Dean, 2016).
526 Despite the growth defect in the benzoate-adapted strain, all three adapted strains produced amino
527 acid labeling similar to the wild type, where the variances in labeling data were generally less than
528 2% (suggesting the invariability of relative flux distributions for biomass synthesis). This provides
529 strong evidence that substrate adaptive evolution did not rewire the central flux network, which is
530 a testament to the rigidity of fluxes during adaptive evolutions (Long et al., 2017).

531

532 **Conclusion and Perspectives**

533 Recently, non-model organisms are gaining attention for addressing challenges in bio-
534 manufacturing from renewable feedstock (Czajka et al., 2017). Researchers leverage innate
535 metabolic strengths of non-model species, including their tolerance and utilization abilities for
536 toxic lignin-derived aromatics. However, rational strain engineering, which is routinely done for
537 model organisms, cannot be done until the complex metabolic networks of these microbes are
538 better understood. In this work, we analyzed the aromatic metabolism in *R. opacus*, one of the

539 promising non-model organisms for lignin bioconversion, using a concerted systems biology
540 approach. Importantly, this is the first reported ^{13}C -metabolic flux analysis completed in any
541 organism using phenol as the sole carbon source. This study also shows that ^{13}C tracing is a
542 powerful tool to precisely elucidate these functional pathways with annotation gaps and to
543 determine the time-scale of metabolic responses. Moreover, we established key links between the
544 transcriptome, metabolome, and fluxome of this organism, and uncovered or confirmed some of
545 its mechanisms for aromatic utilization.

546 By detailing *R. opacus*' metabolic network, this work offers guidelines for developing this
547 species as a bio-manufacturing host. First, phenol-fed *R. opacus* shows strong fluxes through its
548 TCA cycle. Thus, this host is ideal for producing chemicals derived from TCA cycle intermediates
549 (e.g., acetyl-CoA and α -ketoglutarate). In contrast, its fluxes from phenol through gluconeogenesis
550 are small, making it undesirable to redirect sugar phosphate metabolites as precursors for bio-
551 manufacturing. Second, we investigated the carbon source utilization hierarchy of *R. opacus*.
552 Interestingly, this strain utilizes glucose and phenol simultaneously without catabolite repression,
553 preferentially consumes a toxic aromatic compound (phenol) over succinate and acetate, and uses
554 benzoate faster than phenol. Since lignocellulosic degradation products are mixtures of
555 compounds, *R. opacus*'s co-utilization patterns will help to develop predictive metabolic models
556 as well as guide future metabolic engineering efforts toward more-efficient conversion of
557 lignocellulose to biofuels and biochemicals. Combining our findings from this work with our
558 previous reports (Hollinshead et al., 2015; Henson et al., 2018a; Henson et al., 2018b, Yoneda et
559 al., 2016), we are developing a genome-scale metabolic model that enables prediction of metabolic
560 fluxes for diverse carbon sources. Quantitative flux profiles obtained through ^{13}C -MFA provide
561 an accurate description of internal metabolism that is invaluable when deciding future metabolic

562 engineering approaches. For example, these flux profiles can be used in conjunction with methods
563 like ROOM or MOMA to predict the effect of knockouts on metabolism and to offer computer
564 aided strain design (Martín et al., 2015; Ando and Martín, 2018). Third, ^{13}C analysis of the
565 adaptively-evolved strains shows that their genetic differences do not lead to different flux
566 networks. Recently, reliable genetic parts and engineering tools have been developed for *R.*
567 *opacus*, allowing for gene expression control and genome modification in this strain (Delorenzo
568 et al., 2017; Delorenzo et al., 2018). Future work will investigate how model-guided perturbations
569 of its metabolism (e.g., knockouts and overexpression), as opposed to adaptive evolution, affects
570 *R. opacus*'s flux network. With the ^{13}C -MFA model and detailed information regarding *R. opacus*'
571 metabolic network provided from this work, future metabolic engineering efforts will be
572 prediction-driven, speeding up its development as a bio-manufacturing host.

573

574

575 **Data Availability**

576 **1. Supplemental tables and figures**

577 **2. Flux model construction and isotopomer data**

578

579 **Abbreviations**

580 3PG = 3-phosphoglycerate

581 6PG = 6-phosphogluconic acid

582 AcCoA = acetyl-CoA

583 α KG = α -ketoglutarate
584 Asx = aspartate and asparagine
585 Cit = citrate
586 DHAP = dihydroxyacetone phosphate
587 E4P = erythrose-4-phosphate
588 Fum = fumarate
589 F6P = fructose-6-phosphate
590 FBP = fructose-1,6-phosphate
591 G1P = glucose-1-phosphate
592 G1,3P = glycerate-1,3-phosphate
593 G2P = glycerate-2-phosphate
594 G6P = glucose-6-phosphate
595 GAP = glyceraldehyde-3-phosphate
596 Glx = glutamate and glutamine
597 ICit= isocitrate
598 KDPG = 2-dehydro-3-deoxy-phosphogluconate
599 Mal = malate
600 OAC = oxaloacetate
601 PEP = phosphoenolpyruvate
602 Pyr = pyruvate
603 R5P = ribose-5-phosphate
604 Ru5P = ribulose-5-phosphate
605 Suc = succinate
606 SucCoA = succinyl-CoA
607 TA-C3 = 3-carbon fragment transferred by transaldolase
608 TK-C2 = 2-carbon fragment transferred by transketolase
609 X5P=xylose-5-phosphate
610

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615 her help with sample preparation and data analysis of LC-MS experiments.

616

617 Table and Figure Captions

618 **Table 1. A summary of the growth rate, corresponding doubling times (t_D), and maximum**
619 **optical density of *R. opacus* on four different carbon substrates.** All data presented is the
620 average of biological triplicates \pm standard deviation.

621 **Figure 1. The distribution of amino acids (A) and fatty acids (B) in *R. opacus* with three**
622 **different carbon sources.** Asx = aspartate/asparagine and Glx = glutamate/glutamine; fatty acids
623 are denoted as carbon chain length: number of unsaturated bonds, with a B indicating branching.
624 Error bars are estimates of technical uncertainty.

625 **Figure 2. $1\text{-}^{13}\text{C}$ phenol catabolism in *R. opacus* is through the ortho-cleavage.** GC-MS data of
626 alanine gives labeling data for pyruvate. The $[\text{M}-57]^+$ and $[\text{M}-85]^+$ fragments were used to
627 determine the labeling of each carbon of pyruvate. The labeling of pyruvate closely matches the
628 expected labeling for phenol catabolism through the ortho branch. A detailed tracing is shown in
629 **Supplemental Figure 1.**

630 **Figure 3. Flux and transcriptional analyses of *R. opacus* strains.** (A) *R. opacus* PD630's flux
631 map when phenol is the sole carbon source. The flux values are relative flux distributions based
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642 **Figure 4. Dynamic labeling and metabolite pool size measurement.** (A) Pulse-trace labeling
643 data from cultures that were quenched within one minute after ^{13}C phenol was added. Asterisk (*)

644 indicates that the difference between F6P and FBP values is statistically significant ($P < 0.05$, one
645 mean, two-tailed Student's t-test). (B) A simplified metabolic map of the ED and EMP pathways
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648 **Figure 5. Mixotrophic phenol metabolism.** (A) The contribution of phenol-derived carbon to
649 amino acids in the presence of 0.5 g/L U-¹³C phenol and 1 g/L of unlabeled glucose, acetate, or
650 succinate. Samples were taken at early exponential growth ($OD_{600}=0.4\sim0.5$) when both substrates
651 remained in the media. (B) The contribution of phenol to the production of amino acids in the
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653 were taken at early exponential growth phase ($OD_{600}=0.25\sim0.3$) when both substrates remained in
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655 (C) The supernatant concentrations of phenol and benzoate over time when the starting
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660 test) (E) The contribution of acetate to amino acids in the presence of 1 g/L U-¹³C acetate and 1
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665 adapted strain B2, and aromatic-mixture-adapted strain PVHG6; m+n = unlabeled mass m plus n
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