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1 **Characterisation of the hydroxyquinol degradation pathway in *Rhodococcus jostii* RHA1 and**
2 ***Agrobacterium* sp.: an alternative pathway for degradation of protocatechuic acid and lignin**
3 **degradation fragments**

4

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15

16 Running title: Hydroxyquinol pathway in *Rhodococcus jostii* RHA1

17

18 **Abstract:**

19 Deletion of the *pcaHG* genes encoding protocatechuate 3,4-dioxygenase in *Rhodococcus jostii* RHA1
20 gives a gene deletion strain still able to grow on protocatechuic acid as sole carbon source, indicating
21 a second degradation pathway for protocatechuic acid. Metabolite analysis of wild-type *R. jostii*
22 RHA1 grown on media containing vanillin or protocatechuic acid indicated the formation of
23 hydroxyquinol (benzene-1,2,4-triol) as a downstream product. Gene cluster ro01857-ro01860 in
24 *Rhodococcus jostii* RHA1 contains genes encoding hydroxyquinol 1,2-dioxygenase and
25 maleylacetate reductase for degradation of hydroxyquinol but also putative mono-oxygenase
26 (ro01860) and putative decarboxylase (ro01859) genes, and a similar gene cluster is found in the

genome of lignin-degrading *Agrobacterium* sp.. Recombinant *R. jostii* mono-oxygenase and decarboxylase enzymes were in combination found to convert protocatechuic acid to hydroxyquinol. Hence an alternative pathway for degradation of protocatechuic acid via oxidative decarboxylation to hydroxyquinol is proposed.

Importance

There is a well-established paradigm for degradation of protocatechuic acid via the β-ketoadipate pathway in a range of soil bacteria. In this study we have found the existence of a second pathway for degradation of protocatechuic acid in *Rhodococcus jostii* RHA1, via hydroxyquinol (benzene-1,2,4-triol), which establishes a metabolic link between protocatechuic acid and hydroxyquinol. The presence of this pathway in a lignin-degrading *Agrobacterium* sp. strain suggests a possible involvement of the hydroxyquinol pathway in metabolism of lignin degradation fragments.

Introduction

Microbial metabolism of protocatechuic acid (3,4-dihydroxybenzoic acid) via the β-ketoadipate pathway is known to mediate the degradation of many substituted benzoic acids in a range of soil bacteria able to degrade aromatic compounds [1]. The β-ketoadipate pathway has also recently emerged as an important central pathway for degradation of lignin fragments by lignin-degrading bacteria such as *Rhodococcus jostii* RHA1 and *Pseudomonas putida* KT2440 [2], and has been exploited to generate bioproducts from lignin degradation [3-5].

In the course of metabolic engineering studies to generate pyridine-dicarboxylic acid products from lignin degradation via protocatechuic acid [5], a deletion of the *pcaHG* genes encoding protocatechuate 3,4-dioxygenase in *Rhodococcus jostii* RHA1 was made, which revealed to our surprise that this gene deletion mutant was still able to grow on protocatechuic acid as sole carbon source, hence that there is a second pathway for degradation of protocatechuic acid.

Examination of the genome of *Rhodococcus jostii* RHA1 revealed that there is a gene cluster for conversion of hydroxyquinol (benzene-1,2,4-triol) via intradiol oxidative cleavage to maleylacetate, and reduction to β-ketoadipate, encoded by genes ro01857-ro01860 (see Figure 1). The hydroxyquinol catabolic pathway has been observed in *Burkholderia cepacia* AC1100 [6], *Sphingomonas wittichi* RW1 [7], and *Rhodococcus* sp. PN1 [8]. Kasai *et al* have implicated *R. jostii* RHA1 genes ro01857-ro01861 in γ-resorcylate catabolism, via decarboxylation of gamma-resorcylate (2,6-dihydroxybenzoic acid) and hydroxylation of resorcinol to form hydroxyquinol [9]. Transcription of genes ro01857-ro01860 was shown to be regulated by gene regulator TsdR, to which gamma-resorcylate binds as an effector [9]. The oxidative decarboxylation of vanillic acid to 2-methoxyhydroquinone has been reported in extracts of *Sporotrichum pulverulentum* [10], hence the oxidative decarboxylation of protocatechuic acid to form hydroxyquinol has some biochemical precedent. Here we present evidence that conversion of protocatechuic acid to hydroxyquinol occurs in *R. jostii* RHA1, and is mediated by flavin-dependent mono-oxygenase (ro01860) and decarboxylase (ro01859) enzymes.

1

2 Figure 1. Gene cluster (A) and hypothesis (B) for hydroxyquinol pathway in *Rhodococcus jostii*
3 RHA1.

4

5 **Results**

6 *Growth phenotypes of pcaHG gene knockout strain of Rhodococcus jostii RHA1*

7 The *pcaHG* genes encoding protocatechuate 3,4-dioxygenase, the first step on the β-
8 ketoadipate pathway, were deleted in *Rhodococcus jostii* RHA1, using the pk18*mobsacB* plasmid
9 (See Supporting Information Figure S1), containing the *sacB* gene as a counter-selectable marker
10 (Spence EM, Calvo-Bado L, Mines P, Bugg TDH, submitted for publication) [11]. The growth
11 phenotypes of this mutant strains were investigated, using a range of different carbon sources in M9
12 minimal media, as shown in Table 1.

13

14 Table 1. Growth phenotypes of *pcaHG* gene deletion and gene replacement mutant strains.

15

16 As shown in Table 1, the *ΔpcaHG* strain was unable to grow on 0.1% vanillic acid or 4-
17 hydroxybenzoic acid as carbon sources, consistent with their metabolism via the β-ketoadipate
18 pathway, but it was able to grow on solid or liquid M9 media containing 0.1-0.75% (w/v)
19 protocatechuic acid as carbon source (see Supporting Information Figures S2, S3), implying that
20 another pathway could be used by *R. jostii* RHA1 to metabolise protocatechuic acid. The lack of
21 growth on 0.1% vanillic acid, which can be converted to protocatechuic acid, suggests that this
22 alternative pathway is only induced at higher concentrations of protocatechuic acid. Growth on 0.1%
23 protocatechuic acid was accompanied by a dark brown colouration, either on solid or liquid media,
24 which was not observed when the wild-type strain was grown on M9/protocatechuic acid (see
25 Supporting Information Figure S3).

26

1 *Metabolite analysis in Rhodococcus jostii RHA1*

2 Wild-type *R. jostii* RHA1 was grown in either Luria-Bertani broth or M9 minimal media
3 containing 0.1% (w/v) vanillin, vanillic acid, or protocatechuic acid, and after acidification,
4 metabolites formed were extracted into ethyl acetate. The metabolites were then analysed by C_{18}
5 reverse phase HPLC, and by gas chromatography-mass spectrometry (GC-MS), and the observed
6 peaks were compared with authentic standards.

7 The results are shown in Table 2. Consistent with the known vanillic acid degradation
8 pathway [12], vanillin was converted into vanillic acid, and both vanillin and vanillic acid were
9 converted into protocatechuic acid. Vanillin and protocatechuic acid were also converted into a new
10 peak at retention time 11 min which matched an authentic sample of hydroxyquinol, consistent with
11 an oxidative decarboxylation of protocatechuic acid to hydroxyquinol (see Supporting Information
12 Figure S4). Guaiacol was detected as a metabolite of vanillic acid by GC-MS, both in M9 minimal
13 media, and by treatment of vanillic acid with cell extract of *R. jostii* RHA1 in the absence of cofactors,
14 consistent with a decarboxylation of vanillic acid to guaiacol (see Supporting Information Figure S5).
15 When cell-free extract was used in the presence of 0.1 mM NADH, catechol was also observed
16 (Supporting Information Figure S5), but catechol was not observed as a metabolite from
17 protocatechuic acid. Unexpectedly, the formation of vanillic acid as a metabolite was also observed
18 by growth of *R. jostii* on M9 minimal media containing 0.1% protocatechuic acid (Supporting
19 Information Figure S6), implying that methylation of protocatechuic acid can occur.

20

21 Table 2. Metabolites detected by C_{18} reverse phase HPLC or GC-MS

22

23 Growth of the Δ*pcaHG Rhodococcus jostii* gene deletion mutant on M9 minimal media
24 containing protocatechuic acid, as noted above, generated a dark brown coloration. Analysis of
25 supernatant from this incubation by LC-MS at extracted ion *m/z* 127 corresponding to benzene-1,2,4-

triol gave a peak which matched the retention time of a commercial sample of hydroxyquinol (see Figure 2), verifying the conversion of protocatechuic acid into hydroxyquinol.

Figure 2. LC-MS analysis for hydroxyquinol product (extracted ion *m/z* 127).

Investigation of hydroxyquinol gene cluster in Rhodococcus jostii and Agrobacterium sp.

A gene cluster closely related to the ro01857-ro01860 gene cluster of *R. jostii* RHA1 was found in the genome sequence of a lignin-degrading *Agrobacterium* sp. strain identified from municipal waste soil [13]. The *Agrobacterium* sp. gene cluster (genes agro_00119-agro_00125) also contains a putative flavin reductase gene agro_00119 adjacent to flavin mono-oxygenase agro_00120, and an aromatic acid transporter gene (agro_00125). Gene agro_00121 is annotated as a putative decarboxylase (42.7% identity to DBD23_ASPOR *Aspergillus oryzae* 2,3-dihydroxybenzoate decarboxylase), within the PF04909.11 amidohydrolase superfamily. The *Agrobacterium* sp. genome also contained a gene cluster encoding the β-ketoadipate pathway (genes agro_04310-agro_04320), and a DyP-type peroxidase (gene agro_00063), and a putative β-etherase LigE (gene agro_01577), consistent with activity for lignin degradation (see Supporting Information Table S1). Related hydroxyquinol pathway gene clusters are also found on the genomes of *Agrobacterium tumefaciens* F2 (genes Agau_C100398-Agau_C100407), *Agrobacterium* sp. H13-3 (genes AgroH133_08581-AgroH133_08584), and *Rhizobium* species such as *Rhizobium* sp. Kim5 (Kim5_PC00119-Kim5_PC00122).

Figure 3. Comparison of gene clusters for hydroxyquinol utilisation in *Rhodococcus jostii* RHA1 and *Agrobacterium* sp.

The putative mono-oxygenase and decarboxylase genes from *R. jostii* RHA1 and *Agrobacterium* sp. were cloned into expression vector pET-S vector and expressed in *E. coli* BL21(DE3) as N-His_6 fusion proteins. The recombinant mono-oxygenase enzymes Ro01860 and Ag00120 both expressed strongly, and after purification on a Ni-NTA column, gave the expected 60 kDa protein bands by SDS-PAGE (see Supporting Information Figure S7). The putative decarboxylase enzymes Ro01859 and Ag00121 both expressed weakly, giving weaker bands by SDS-PAGE corresponding to the expected 44.7 and 50.1 kDa proteins respectively (see Supporting Information Figure S7). Western blots were carried out in order to confirm that the desired decarboxylase protein had been expressed, which confirmed that a protein of the correct size had been expressed at low levels (see Supporting Information Figure S8).

The purified recombinant enzymes were incubated with protocatechuic acid, in the presence of 10 μM FAD and 200 μM NADPH, in order to test for conversion to hydroxyquinol, and the reactions monitored by C_{18} reverse phase HPLC. Incubation of 1 mM protocatechuic acid with 100 μg purified Ro01860 and Ro01859 enzymes gave no new product peak after 1 hr, however, addition of 100 μg purified mono-oxygenase Ro01860 and cell extract containing recombinant decarboxylase Ro01859 (100 μg protein) to 1 mM protocatechuic acid was found to generate a new peak at 10.7 min corresponding to hydroxyquinol (see Figure 4A), but this peak was not formed by cell extract lacking Ro01859. Using 100 μg recombinant mono-oxygenase Ro01860 or Ag00120 alone, some consumption of protocatechuic acid was observed (35% & 77% respectively), but hydroxyquinol was not formed, although a new peak at retention time 12.8 min was generated which did not co-elute with hydroxyquinol (see Supporting Information Figure S9). Using purified recombinant decarboxylase enzymes Ro01859 or Ag00121 alone, no reaction with protocatechuic acid was observed. We hypothesise that purification of decarboxylase Ro01859 renders the enzyme inactive, perhaps due to loss of an essential cofactor, but that overexpressed decarboxylase Ro01859 and purified mono-oxygenase Ro01860 are together able to catalyse the conversion of protocatechuic acid to hydroxyquinol.

Figure 4. Reverse phase HPLC analysis of incubations of (A) protocatechuic acid (PCA) (B) catechol and (C) hydroquinone (1,4-HQ) (D) gentisic acid with purified mono-oxygenase Ro01860 and overexpressed decarboxylase Ro01859.

Several other potential substrates for the mono-oxygenase and decarboxylase enzymes were also tested. Formation of hydroxyquinol was also observed upon incubation of Ro01859/Ro01860 with catechol (see Figure 4B), 1,4-hydroquinone (see Figure 4C), or gentisic acid (2,5-dihydroxybenzoic acid, see Figure 4D), indicating that hydroxylation/decarboxylation of several compounds can be carried out by this pair of enzymes *in vitro*, as discussed below. The formation of a small amount of hydroxyquinol was also observed when catechol was treated with mono-oxygenase Ro01860 alone, approximately 2% of the product formed with Ro01859/Ro01860. No reaction of Ro01860 alone was observed with 1,4-hydroquinone, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, or gentisic acid (2,5-dihydroxybenzoic acid).

Discussion

The hydroxyquinol degradation pathway has been previously reported in *Burkholderia cepacia* AC1100 [6], *Sphingomonas wittichi* RW1 [7], and *Rhodococcus sp.* PN1 [8], as an aromatic catabolic pathway, and a hydroxyquinol degradation gene cluster has been observed in *Cupriavidus necator* JMP134 [14]. This pathway has been implicated in degradation of 4-nitrophenol in *Arthrobacter chlorophenolicus* A6 [15], and in γ-resorcylate catabolism in *R. jostii* RHA1 [9]. Following our initial observation that a Δ*pcaHG* gene deletion mutant of *R. jostii* RHA1 was able to grow on minimal media containing protocatechuic acid, we hypothesised that protocatechuic acid could be converted via flavin-dependent mono-oxygenase (ro01860) and a decarboxylase (ro01859) into hydroxyquinol. We have shown by metabolite analysis that protocatechuic acid can be converted into hydroxyquinol by wild-type *R. jostii* RHA1 cells, by the Δ*pcaHG* gene deletion strain, and *in*

vitro using recombinant Ro01860/Ro01859 enzymes. These observations therefore establish a metabolic link between protocatechuic acid and hydroxyquinol in *R. jostii*, which can be used as an alternative pathway for metabolism of protocatechuic acid in this bacterium.

The catalytic mechanism of these two enzymes is likely to proceed as shown in Figure 5. Phenol hydroxylation *ortho-* or *para-* to a phenolic hydroxyl group is well precedented in the flavin mono-oxygenase family, via a flavin hydroperoxide reaction intermediate [16]. Several soil bacteria that can metabolise chlorinated phenols [17-19] or 4-nitrophenols [20,21] are known to express flavin-dependent mono-oxygenase enzymes that catalyse hydroxylation *para* to a phenolic hydroxyl group, followed by loss of either a 4-chloro or 4-nitro substituent. In this case, hydroxylation in the 1-position would be catalysed by mono-oxygenase Ro01860, to give a diffusible intermediate, which is then decarboxylated by Ro01859, using the carbonyl group at C-4 as an electron sink to aid decarboxylation (see Figure 5). Such a mechanism would rationalise why a combination of both Ro01860 and Ro01859 are needed in order to effect this oxidative decarboxylation, and why no activity was observed using decarboxylase Ro01859 alone. Using mono-oxygenase Ro01860 alone the intermediate would be formed, leading to consumption of protocatechuic acid as observed, but the intermediate is likely to be chemically unstable, and could not be characterised.

Figure 5. Proposed catalytic mechanism for mono-oxygenase Ro01860 and decarboxylase Ro01859

Formation of hydroxyquinol *in vitro* was also observed from catechol, 1,4-hydroquinone, or 2,5-dihydroxybenzoic acid (gentisic acid). Only a small amount of hydroxylation of catechol by mono-oxygenase Ro01860 alone was observed, implying that the two enzymes are more active in combination, perhaps forming a complex. Kasai *et al* have shown that the *R. jostii* ro01859 and ro01860 gene products are able to convert 2,6-dihydroxybenzoic acid to hydroxyquinol: Ro01859 decarboxylates γ-resorcylate to resorcinol, and Ro01860 converts resorcinol to hydroxyquinol [9]. Hence it appears that these enzymes are able to catalyse several hydroxylation/decarboxylation

1 reactions. Kasai *et al* reported that gene regulator TsdR negatively regulates the expression of these
2 genes, and binds to γ-resorcylic acid (2,6-dihydroxybenzoic acid). The observation that the Δ*pcaHG*
3 deletion mutant can grow on M9/0.1% protocatechuic acid but not on 0.1% vanillic acid or 0.1% 4-
4 hydroxybenzoic acid suggests that the conversion of protocatechuic acid to hydroxyquinol takes place
5 only at higher concentrations of protocatechuic acid, hence protocatechuic acid may bind more
6 weakly to gene regulator TsdR. Kasai *et al* reported activity for resorcinol hydroxylation by Ro01860
7 using NADH as cofactor [9], whereas we observed activity for protocatechuic acid conversion by
8 Ro01860 and Ro01859 using NADPH as cofactor (NADH not tested), therefore it appears that
9 Ro01860 can accept either NADH or NADPH for different transformations.
10 Our metabolite analysis also indicates two other transformations that can occur in *R. jostii*
11 RHA1, as shown in Figure 6. Firstly, we have observed the conversion of vanillic acid to guaiacol
12 via decarboxylation, followed by demethylation to catechol. Decarboxylation of vanillic acid to
13 guaiacol is precedented in *Bacillus megaterium* and *Streptomyces* [22], a gene cluster responsible for
14 this transformation has been identified in *Streptomyces* sp. D7 [23], and a vanillate decarboxylase
15 enzymes has been purified from *Nocardia* sp. NRRL 5646 [24], however, there is no vanillic acid
16 decarboxylase gene annotated on the *R. jostii* RHA1 genome, so the gene responsible for this
17 transformation is not known. The decarboxylation of vanillate to guaiacol might explain an earlier
18 observation where a *R. jostii* vanillate mono-oxygenase (ro04165) gene deletion strain failed to
19 accumulate vanillic acid when grown on media containing wheat straw lignocellulose, whereas a
20 Δ*vdh* vanillate dehydrogenase gene deletion strain did accumulate vanillin [3]. We did not observe
21 any conversion of protocatechuic acid to catechol, and there is no protocatechuic acid decarboxylase
22 gene annotated on the *R. jostii* RHA1 genome, though such a decarboxylase gene has been identified
23 in *Klebsiella pneumoniae* [25]. The formation of guaiacol is also consistent with the presence of a
24 gene cluster for guaiacol metabolism in *R. jostii* RHA1 at ro08067/08068, containing a homologue
25 for a P450-dependent guaiacol demethylase enzyme which has been characterised from
26 *Amycolatopsis* sp. ATCC 39116 [26].

1

2 Figure 6. Biochemical transformations of vanillic acid and protocatechuic acid in *Rhodococcus jostii*
3 RHA1 observed in this work, and the transformation reported by Kasai et al [9].

4

5 Secondly, a surprising observation was the conversion of protocatechuic acid to vanillic acid,
6 via methylation of the 3-hydroxy group. There is a methyltransferase gene (ro04166) located in the
7 gene cluster for vanillate mono-oxygenase (ro04165), which may be responsible for this methylation
8 reaction. This reaction is surprising, as it would apparently set up a "futile cycle" between vanillic
9 acid and protocatechuic acid, therefore expression of the methyltransferase and demethylase genes
10 would need to be tightly regulated.

11 We have elsewhere observed 2-methoxyhydroquinone, the methylated form of
12 hydroxyquinol, as a metabolite of lignin oxidation by lignin-degrading enzyme manganese
13 superoxide dismutase from *Sphingobacterium* sp T2 [27]. A pathway for degradation of (+)-
14 pinoresinol, a component of lignin structure, via 2-methoxyhydroquinone has also recently been
15 elucidated in *Pseudomonas* sp. SG-MS2 [28,29]. Hence it seems likely that hydroxyquinol can be
16 generated from other lignin oxidation reactions involving aryl-Cα oxidative cleavage of the aryl-C_3
17 unit found in lignin. In a recent survey of the genomes of lignin-degrading bacteria [30], the
18 hydroxyquinol gene cluster was only observed in *Rhodococcus jostii* RHA1 and *Agrobacterium* sp.,
19 but lignin-degrading *Paenibacillus* sp. and *Ochrobactrum* sp. strains also contained *mhq* genes
20 thought to be involved in hydroquinone breakdown [31]. The pathways branching from vanillic acid
21 and protocatechuic acid shown in Figure 6 will reduce metabolic flux through the β-ketoadipate
22 pathway, and hence an understanding of these pathways will underpin efforts to engineer *R. jostii*
23 RHA1 for efficient conversion of lignin to renewable chemicals [3-5].

24

25 **Materials & Methods**

26 *Bacterial strains, plasmids and chemicals*

Rhodococcus jostii RHA1 was used as the ancestral strain [32]. Δ*pcaHG R. jostii* contains a deletion in chromosomal *pcaHG* genes, as described herein. *Agrobacterium* sp. was isolated from municipal waste as described previously [13]. For routine growth and maintenance, *R. jostii* or *Agrobacterium* sp. cells were cultured in liquid or solid Luria-Bertani broth (LB) medium, or for *R. jostii* on M9 minimal media supplemented with 0.1% benzoic acid, at 30 °C and with shaking at 180 rpm if required. Bacterial growth was measured by absorbance at 595 or 600 nm.

Expression vector pET-S is a derivative of pET151 topo (Invitrogen) in which a Sumo tag was amplified from pOPINS3C (http://www.oppf.rc-harwell.ac.uk/OPPF/protocols/cloning.jsp) using primers P_{for} CCCCCCCATATGGCACACCATCACCACC (NdeI site) and P_{rev} AAAAAAATCGATGGATTTAAATGGGCTAGCGGATCCACCGCTGCTGATCTGTTCG (ClaI, SwaI, NheI, BamHI sites), and cloned into NdeI/ClaI sites of pET151 topo. All chemicals were purchased form Sigma Aldrich unless otherwise stated.

Construction of gene deletion mutants.

The Δ*pcaHG* gene deletion strain was constructed using the pk18*mobsacB* plasmid, which uses the *sacB* gene (confers sucrose sensitivity) as a counter-selectable marker [11]. PCR was used to amplify two 1 kb regions of chromosomal DNA on either side of the genes to be deleted. The amplified 1 kb upstream and downstream DNA sequences were ligated into pk18*mobsac* and the resulting construct (see Supporting Information Figure S1) was confirmed by DNA sequencing and restriction digestion. The recombinant plasmid was taken up into *R. jostii* by electroporation, and recombinant colonies selected by kanamycin resistance. Isolation of the double cross-over gene deletion was carried out using sucrose resistance counter-selection [11], and the markerless gene deletion was confirmed by internal and external PCR analysis.

Metabolite analysis.

1 Cultures of *R. jostii* RHA1 were grown in either Luria-Bertani media or M9 minimal media
2 containing 1 mM vanillic acid, vanillin or protocatechuic acid at 30 °C for 24-48 hr. 500 µL aliquots
3 of culture were removed and combined with 500 µL of HPLC grade methanol/0.1% trifluoroacetic
4 acid. Samples were vortexed and then centrifuged (microcentrifuge) for 15 minutes.
5 Biotransformations using *R. jostii* cell-free extract contained 100 µg protein, 1 mM substrate, with or
6 without addition of 0.1 mM NADH, in 50 mM Tris buffer pH 7.5, and were incubated at 20 °C for 1-
7 2 hours. The incubation was then acidified to pH 1 (1M HCl), and the products extracted into ethyl
8 acetate, solvent evaporated at reduced pressure, and the sample re-dissolved in methanol. HPLC
9 analysis of the supernatant was performed using a Hyperclone 5 µm C_{18} reverse phase column
10 (Phenomenex), using an Agilent 1200 series HPLC analyser. The HPLC solvents were water/0.1%
11 trifluoracetic acid (solvent A) and methanol/0.1% trifluoracetic acid (solvent B). The applied gradient
12 was 10-30 % B over 5 min; 30-40 % B over 15 min; 40-70 % B over 10 min; 70-100 % B over 5 min;
13 100-10 % B over 10 min, at a flow rate of 0.5 ml min^{-1}. UV detection was at 270 nm. Samples for
14 LC-MS analysis were suspended in 1:1 MeOH/water, and were separated on a Phenomenex Luna C_{18}
15 column (5 µm, 100 Å, 50 mm, 4.6 mm) on an Agilent 1200 analyzer and Bruker HCT Ultra mass
16 spectrometer, flow rate 0.5 mL/min, monitoring at 270 nm. Solvent A water/ 0.1% formic acid,
17 solvent B MeOH/0.1% formic acid. Gradient: 15% solvent B for 5 min; 15-25% B from 5-15 min;
18 25-70% B from 15-23 min; 70-100% from 23-30 min. GC-MS analysis (ion-trap analyzer) was
19 performed using a Varian 4000 gas chromatograph-mass Spectrometer, with a Varian Factor Four
20 column (length 30m, i.d 0.25 mm, thickness 0.25 µm). Electron-impact mass spectra (EI-MS) were
21 recorded at an ionization energy of 70eV.
22
23 *Expression & purification of R. jostii and Agrobacterium mono-oxygenase and decarboxylase*
24 *enzymes*
25 *R. jostii* ro01859 (decarboxylase) and ro01860 (mono-oxygenase) genes and *Agrobacterium*
26 agro_00120 (mono-oxygenase) and agro_00121 (decarboxylase) genes were amplified from genomic

1 DNA using polymerase chain reaction, using the oligonucleotide primers given below, and ligated
2 into the pET-S expression vector, using XbaI and ClaI restriction sites, resulting in the expression of
3 an N-His_6 fusion protein. Oligonucleotide primers for PCR:
4 ro01859: forward AAAAAAGCTAGCCAGGGCAAGATCGCACTGG;
5 reverse AAAAAAATCGATTCACCGGTCGAGTTTGAACAAC
6 ro01860: forward AAAAAAAGCTAGCATGTCTGCCTTCGCACAG;
7 reverse AAAAAAATCGATAGGTGTTTGTCGGTGGTG
8 ag00120: forward AAAAAAAGCTAGCATGAACGATATGAGCCATGCG;
9 reverse AAAAAAATCGATTCAATATTGGCCCTTGGGTTC
10 ag00121: forward AAAAAAAGCTAGCATGCAAGGCAAGGTCGCTC;
11 reverse AAAAAAATCGATTCAGTTCCCGTCGAGTTTGAAC.
12 Recombinant plasmids were expressed in *E. coli* BL21(DE3). For protein purification,
13 constructs were grown in Luria Bertani broth (500 mL) in the presence of ampicillin (50 μg/mL), and
14 protein expression induced by addition of IPTG (250 μM) at OD_{600} = 0.6. Expression of mono-
15 oxygenases Ro01860 and Ag00120 was carried out the presence of 1 mM riboflavin, in order to
16 achieve reconstitution of the flavin cofactor. Cultures were grown for 16 hr at 16 ^{o}C, and then cells
17 harvested by centrifugation (4,000 g, 20 min). Cells were resuspended in 50 mM sodium phosphate
18 buffer pH 8.0 containing 300 mM NaCl, and sonicated in a Constant Systems Cell Disruptor (20000
19 psi), then cell debris removed by centrifugation (20,000 g, 20 min). Cell extract was applied to a His
20 Gravitrap column (GE Healthcare), washed with 50 mM sodium phosphate buffer pH 8.0 containing
21 300 mM NaCl and 20 mM imidazole, and then protein eluted with 50 mM sodium phosphate buffer
22 pH 8.0 containing 300 mM NaCl and 250 mM imidazole. Purified protein was then desalted using a
23 PD-10 desalting column (GE Healthcare).
24 SDS-PAGE was carried out using a Thermo Fisher Scientific Invitrogen Mini Gell Tank,
25 using a 10% Bis-Tris protein gel, according to manufacturer's instructions. Thermo Fisher Scientific
26 PageRuler prestained protein ladder was used for molecular weight markers. Western bolt was carried

1 on using a Thermo Fisher Scientific InvitrogenTM NovexTM iBlotTM 2 Nitrocellulose Transfer
2 Stack and a Thermo Fisher Scientific iBlotTM 2 Gel Transfer Device, according to manufacturer's
3 instructions, using Thermo Fisher Scientific 6x-His Tag Monoclonal Antibody.
4
5 *Biotransformation using mono-oxygenase Ro01860 and decarboxylase Ro01859*
6 Biotransformations using recombinant Ro01860 and Ro01859 were carried out using 100 µg
7 of each protein, in PBS buffer (1.0 mL) containing 1 mM substrate, in the presence of 10 µM FAD
8 and 200 µM NADPH, incubated for 60 min at 25 ^{o}C. Aliquots (100 µL) were removed and combined
9 with 100 µL of HPLC grade methanol/0.1% trifluoracetic acid. Samples were vortexed and then
10 centrifuged (microcentrifuge) for 15 minutes, and analysed by HPLC using a Zorbax Eclipse plus
11 (Agilent) C_{18} reverse phase column, using the gradient and conditions described above. Control
12 incubations lacking enzyme were also carried out, which showed no substrate conversion.
13
14 **Accession numbers**
15 *R. jostii* enzymes: ro01859 decarboxylase ABG93670; ro01860 flavin-dependent mono-oxygenase
16 ABG93671. *Agrobacterium* sp. enzymes: agro_00120 flavin-dependent mono-
17 oxygenase WP_149145897.1; agro_00121 decarboxylase WP_149145898.1.
18
19
20 **Acknowledgements**
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24
25 **References**

1. Harwood CS, Parales RE. 1996. The β-ketoadipate pathway and the biology of self-identity. Annu Rev Microbiol 50:553-590.

2. Bugg TDH, Ahmad M, Hardiman EM, Rahmanpour R. 2011. Pathways for degradation of lignin in bacteria and fungi. Nat Prod Reports 28:1883-1896.

3. Sainsbury PD, Hardiman EM, Ahmad M, Otani H, Seghezzi N, Eltis LD, Bugg TDH. 2013. Breaking down lignin to high-value chemicals: the conversion of lignocellulose to vanillin in a gene deletion mutant of *Rhodococcus jostii* RHA1. ACS Chem Biol 8:2151-2156.

4. Vardon DR, Franden MA, Johnson CW, Karp EM, Guarneri MT, Linger JG, Salm MJ, Strathman TJ, Beckham GT. 2015. Adipic acid production from lignin. Energy Environ Sci 8:617-628.

5. Mycroft Z, Gomis M, Mines P, Law P, Bugg TDH. 2015. Biocatalytic conversion of lignin to aromatic dicarboxylic acids in *Rhodococcus jostii* RHA1 by re-routing aromatic degradation pathways. Green Chemistry 17:4974-4979.

6. Daubaras DL, Saido K, Chakrabarty AM. 1996. Purification of hydroxyquinol 1,2-dioxygenase and maleylacetate reductase: the lower pathway of 2,4,5-trichlorophenoxyacetic acid metabolism by *Burkholderia cepacia* AC1100. Appl Environ Microbiol 62:4276 – 4279.

7. Armengaud J, Timmis KN, Wittich RM. 1999. A functional 4-hydroxy- salicylate/hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-*p*-dioxin pathway genes in *Sphingomonas* sp. strain RW1. J Bacteriol 181:3452–3461.

8. Yamamoto K, Nishimura M, Kato D, Takeo M, Negoro S. 2011. Identification and characterization of another 4-nitrophenol degradation gene cluster, *nps*, in *Rhodococcus* sp. strain PN1. J Biosci Bioeng 111:687– 694.

9. Kasai D, Araki N, Motoi K, Yoshikawa S, Iino T, Imai S, Masai E, Fukuda M. 2015. γ-Resorcylate catabolic-pathway genes in the soil actinomycete *Rhodococcus jostii* RHA1. Appl Environ Microbiol 81:7656 –7665.

1 10. Buswell JA, Ander P, Pettersson B, Eriksson K. 1979. Oxidative decarboxylation of vanillic acid
2 by *Sporotrichum pulverulentum*. FEBS Lett 103:98-101.
3 11. van der Geize R, Hessels GI, van Gerwen R, van der Meijden P, Dijkhuizen L 2001. Unmarked
4 gene deletion mutagenesis of *kstD*, encoding 3-ketosteroid-D1-dehydrogenase, in *Rhodococcus*
5 *erythropolis* SQ1 using *sacB* as a counter-selectable marker. FEMS Microbiol Lett 205:197-202.
6 12. Chen HP, Chow M, Liu CC, Lau A, Liu J, Eltis LD 2012. Vanillin catabolism in *Rhodococcus*
7 *jostii* RHA1. Appl Environ Microbiol 78:586-588.
8 13. Rashid GMM, Duran-Pena MJ, Rahmanpour R, Sapsford D, Bugg TDH. 2017. Delignification
9 and enhanced gas release from soil containing lignocellulose by treatment with bacterial lignin
10 degraders. J Appl Microbiol 123:159-171.
11 14. Pérez-Pantoja D, De la Iglesia R, Pieper DH, González B. 2008. Metabolic reconstruction of
12 aromatic compound degradation from the genome of the amazing pollutant-degrading bacterium
13 *Cupriavidus necator* JMP134. FEMS Microbiol Rev 32:736-794.
14 15. Nordin K, Unell M, and Jansson JK. 2005. Novel 4-chlorophenol degradation gene cluster and
15 degradation route via hydroxyquinol in *Arthrobacter chlorophenolicus* A6. Appl Environ Microbiol
16 71:6538-6544.
17 16. Massey V. 1994. Activation of molecular oxygen by flavins and flavoproteins. J Biol Chem
18 269:22459-22462.
19 17. Beadle CA, Smith ARW. 1982. The purification and properties of 2,4-dichlorophenol
20 hydroxylase from a strain of *Acinetobacter* species. Eur J Biochem 123:323-332.
21 18. Xun L, Orser CS. 1991. Purification and properties of pentachlorophenol hydroxylase, a
22 flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J Bacteriol 173:4447-4453.
23 19. Wieser M, Wagner B, Eberspächer J, Lingens F. 1997. Purification and characterization of 2,4,6-
24 trichlorophenol-4-monooxygenase, a dehalogenating enzyme from *Azotobacter* sp. strain GP1. J
25 Bacteriol 179:202-208.

1 20. Leungsakul T, Johnson GR, Wood TK. 2006. Protein engineering of the 4-methyl-5-nitrocatechol
2 monooxygenase from *Burkholderia* sp. strain DNT for enhanced degradation of nitroaromatics. Appl
3 Environ Microbiol 72:3933-3939.
4 21. Min J, Lu Y, Hu X, Zhou N-Y. 2016. Biochemical characterization of 3-methyl-4-nitrophenol
5 degradation in *Burkholderia* sp. strain SJ98. Front Microbiol 7:791.
6 22. Crawford RL, Olson PP. 1978. Microbial catabolism of vanillate: decarboxylation to guaiacol.
7 Appl Environ Microbiol 36:539-543.
8 23. Chow KT, Pope MK, Davies J. 1999. Characterization of a vanillic acid non-oxidative
9 decarboxylation gene cluster from *Streptomyces* sp. D7. Microbiology 145:2393-2403.
10 24. Dhar A, Lee K-S, Dhar K, Rosazza JPN. 2007. *Nocardia* sp. vanillic acid decarboxylase. Enz
11 Microb Technol 41:271-277.
12 25. Sonoki T, Morooka M, Sakamoto K, Otsuka Y, Nakamura M, Jellison J, Goodell B. 2014.
13 Enhancement of protocatechuate decarboxylase activity for the effective production of muconate
14 from lignin-related aromatic compounds. J Biotechnol 192:71-77.
15 26. Mallinson SJB, Machinova MM, Silveira RL, Garcia-Borras M, Gallup N, Johnson CW, Allen
16 MD, Skaf MS, Crowley MF, Neidle EL, Houk KN, Beckham GT, DuBois JL, McGeehan JE. 2018.
17 A promiscuous cytochrome P450 aromatic *O*-demethylase for lignin bioconversion. Nature Commun
18 9:2487
19 27. Rashid GMM, Taylor CR, Liu Y, Zhang X, Rea D, Fülöp V, Bugg TDH. 2015. Identification of
20 manganese superoxide dismutase from *Sphingobacterium sp*. T2 as a novel bacterial enzyme for
21 lignin oxidation. ACS Chem Biol 10:2286-2294.
22 28. Shettigar M, Balotra S, Cahill D, Warden AC, Lacey MJ, Kohler HPE, Rentsch D, Oakeshott JG,
23 Pandey G. 2018. Isolation of the (+)-pinoresinol-mineralizing *Pseudomonas* sp. strain SG-MS2 and
24 elucidation of its catabolic pathway. Appl Environ Microbiol 84:e02531-17.
25 29. Shettigar M, Balotra S, Kasprzak A, Pearce SL, Lacey MJ, Taylor MC, Liu JW, Cahill D,
26 Oakeshott JG, Pandey G. 2020. Oxidative catabolism of (+)-pinoresinol is initiated by an unusual

flavocytochrome encoded by translationally coupled genes within a cluster of (+)-pinoresinol-coinduced genes in *Pseudomonas* sp. strain SG-MS2. Appl Environ Microbiol 86:e00375-20.

30. Granja-Travez RS, Persinoti GF, Squina FM, and Bugg TDH. 2020. Functional genomic analysis of bacterial lignin degraders: diversity in mechanisms of lignin oxidation and metabolism. Appl Microbiol Biotechnol 104:3305-3320.

31. Tago K, Sato J, Takesa H, Kawagishi H, Hayatsu M. 2005. Characterization of methylhydroquinone-metabolizing oxygenase genes encoded on plasmid in *Burkholderia* sp. NF100. J Biosci Bioeng 100:517–523.

32. Seto, M, Kimbara K, Shimura M, Hatta T, Fukuda M, Yano K. 1995. A novel transformation of polychlorinated biphenyls by *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. 61:3353–3358.

Construct	M9/vanillic acid	M9/vanillin	M9/4-hydroxy-benzoic acid	M9/ferulic acid	M9/proto-catechuic acid
Wild-type *R. jostii* RHA1	+++	++	+++	+++	+++
ΔpcaHG	-	-	-	-	+

Table 1. Growth phenotypes of wild-type *R. jostii* RHA1 and Δ*pcaHG* gene deletion strains, grown with 0.1% carbon source in liquid M9 minimal media. Phenotypes: +++, strong growth ($OD_{600} > 0.6$) after 48 hr; ++, growth (OD_{600} 0.3-0.6) after 48 hr; +, weak growth (OD_{600} 0.2-0.3) after 48 hr; -, no growth. OD_{600} 0.15-0.2 at start of culture.

Substrate	Media	Metabolites observed				
		Vanillic acid	Protocatechuic acid	Hydroxyquinol	Guaiacol	Catechol
Vanillin	LB	++	++	+		
	M9	++				
Vanillic acid	LB		++			
	M9				+ (GC)	
	Extract[a]				+ (GC)	
	+ NADH[b]		++			+
Protocatechuic acid	M9	+		+		

Table 2. Metabolites detected by C_{18} reverse phase HPLC or GC-MS after growth of *R. jostii* RHA1 in either Luria-Bertani broth (LB) or M9 minimal media containing 0.1% (w/v) carbon source, or treatment with *R. jostii* RHA1 cell extract. Key: +, metabolite observed; ++, strong formation of metabolite; GC, metabolite detected by GC-MS; a, cell-free extract contained 100 µg protein in 50 mM Tris buffer pH 7.5 (5 mL); b, with addition of 0.1 mM NADH. Retention times and gradients are described in Materials & Methods section.

Figure Legends

Figure 1. Gene cluster (A) and hypothesis (B) for hydroxyquinol pathway in *Rhodococcus jostii* RHA1.

Figure 2. LC-MS analysis for hydroxyquinol product (extracted ion *m/z* 127). A. Analysis of incubation of Δ*pcaHG R. jostii* deletion mutant with protocatechuic acid. B. Analysis of commercial sample of hydroxyquinol (benzene-1,2,4-triol).

Figure 3. Comparison of gene clusters for hydroxyquinol utilisation in *Rhodococcus jostii* RHA1 and *Agrobacterium* sp.

Figure 4. Reverse phase HPLC analysis of incubations of (A) protocatechuic acid (PCA), (B) catechol, (C) hydroquinone (1,4-HQ), and (D) gentisic acid with purified mono-oxygenase Ro01860 and overexpressed decarboxylase Ro01859. Biotransformations contained 100 μg of each protein, in PBS buffer (1.0 mL), containing 1 mM substrate, 10 μM FAD and 200 μM NADPH, and were incubated for 60 min at 25 °C. Hydroxyquinol product is marked with blue box, other substrates are labelled, gentisic acid substrate elutes at >15 min.

Figure 5. Proposed catalytic mechanism for mono-oxygenase Ro01860 and decarboxylase Ro01859

Figure 6. Biochemical transformations of vanillic acid and protocatechuic acid in *Rhodococcus jostii* RHA1 observed in this work, and the transformation reported by Kasai et al [9].

A.

ro01857 ro01858 ro01859 ro01860

intradiol catechol dioxygenase | maleylacetate reductase | decarboxylase | flavin-dependent mono-oxygenase

B.

CO_2H OH OH

decarboxylase ro01859

mono-oxygenase ro01860

OH OH OH hydroxyquinol

intradiol catechol dioxygenase ro01857

OH CO_2H CO_2H

protocatechuate 3,4-dioxygenase (pcaHG)

CO_2H CO_2H CO_2H

β-ketoadipate pathway

O CO_2H CO_2H β-ketoadipate

maleylacetate reductase

NADH

O CO_2H CO_2H

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2 Figure 1

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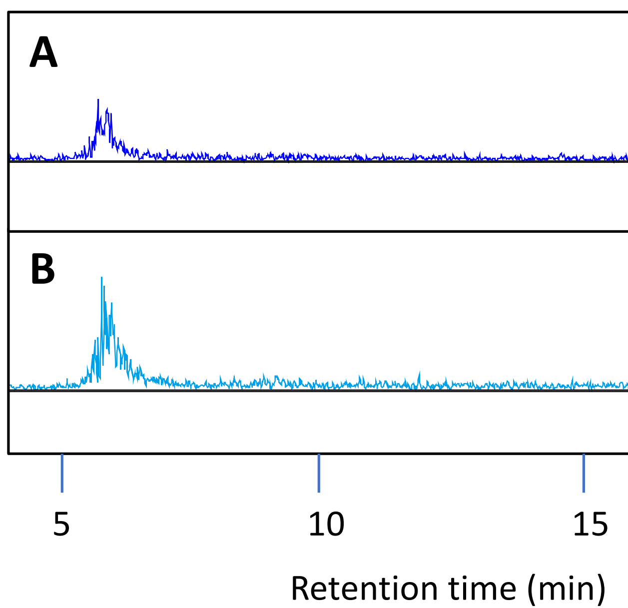

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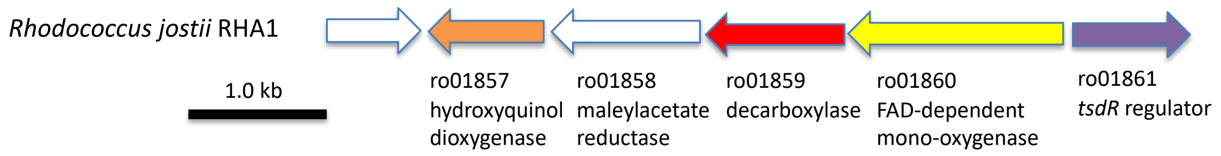

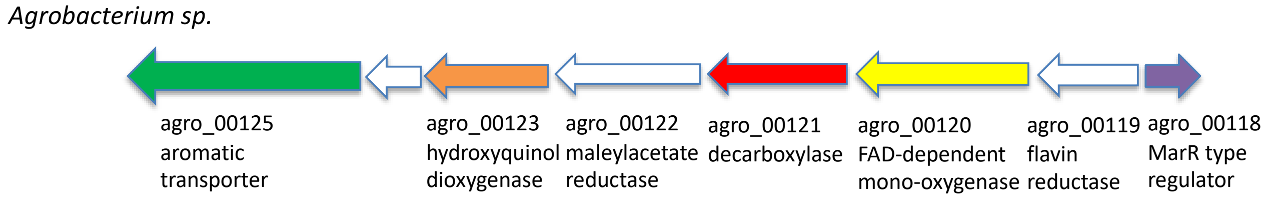

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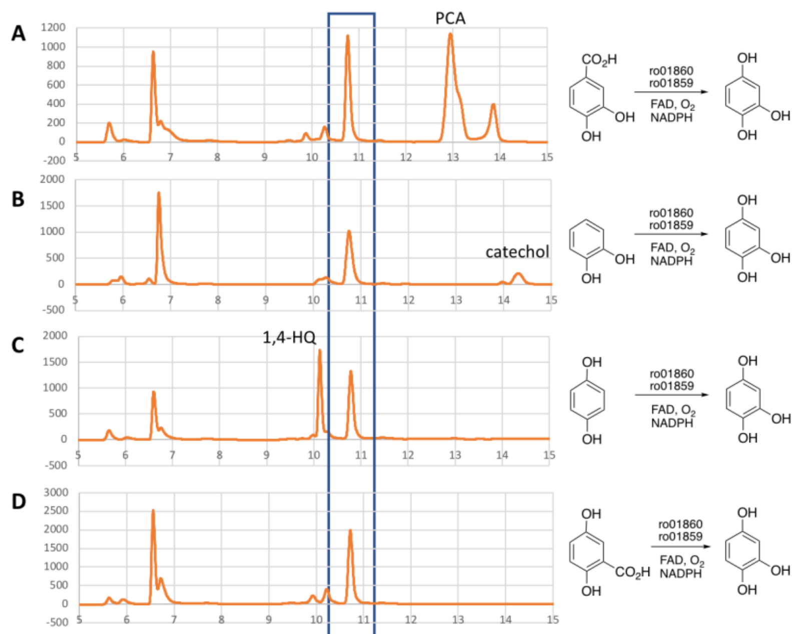

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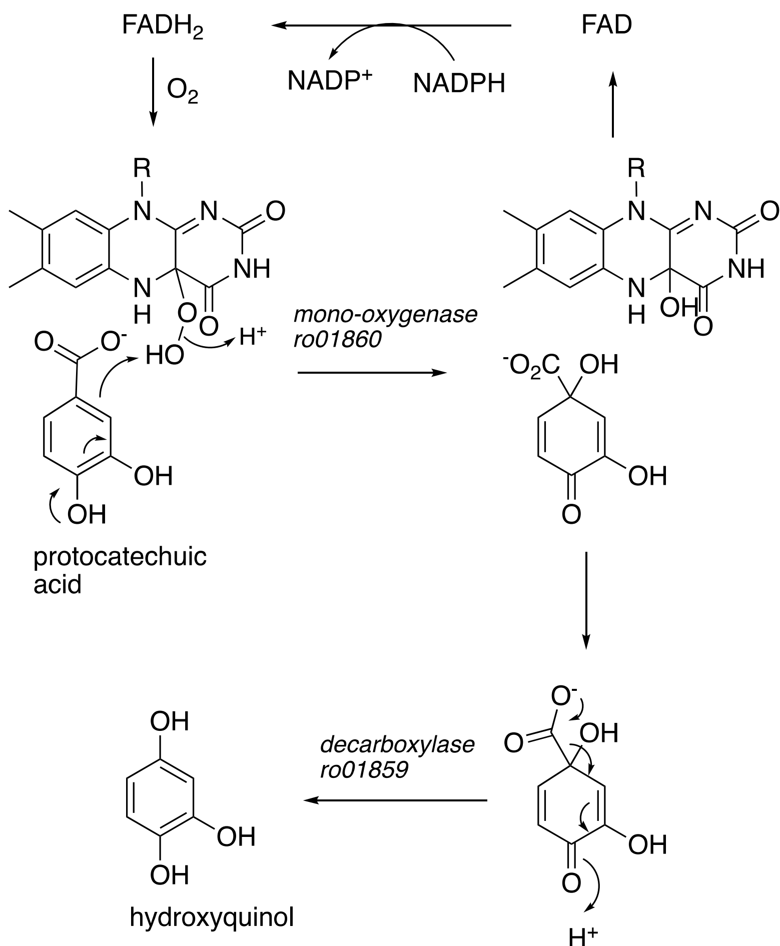

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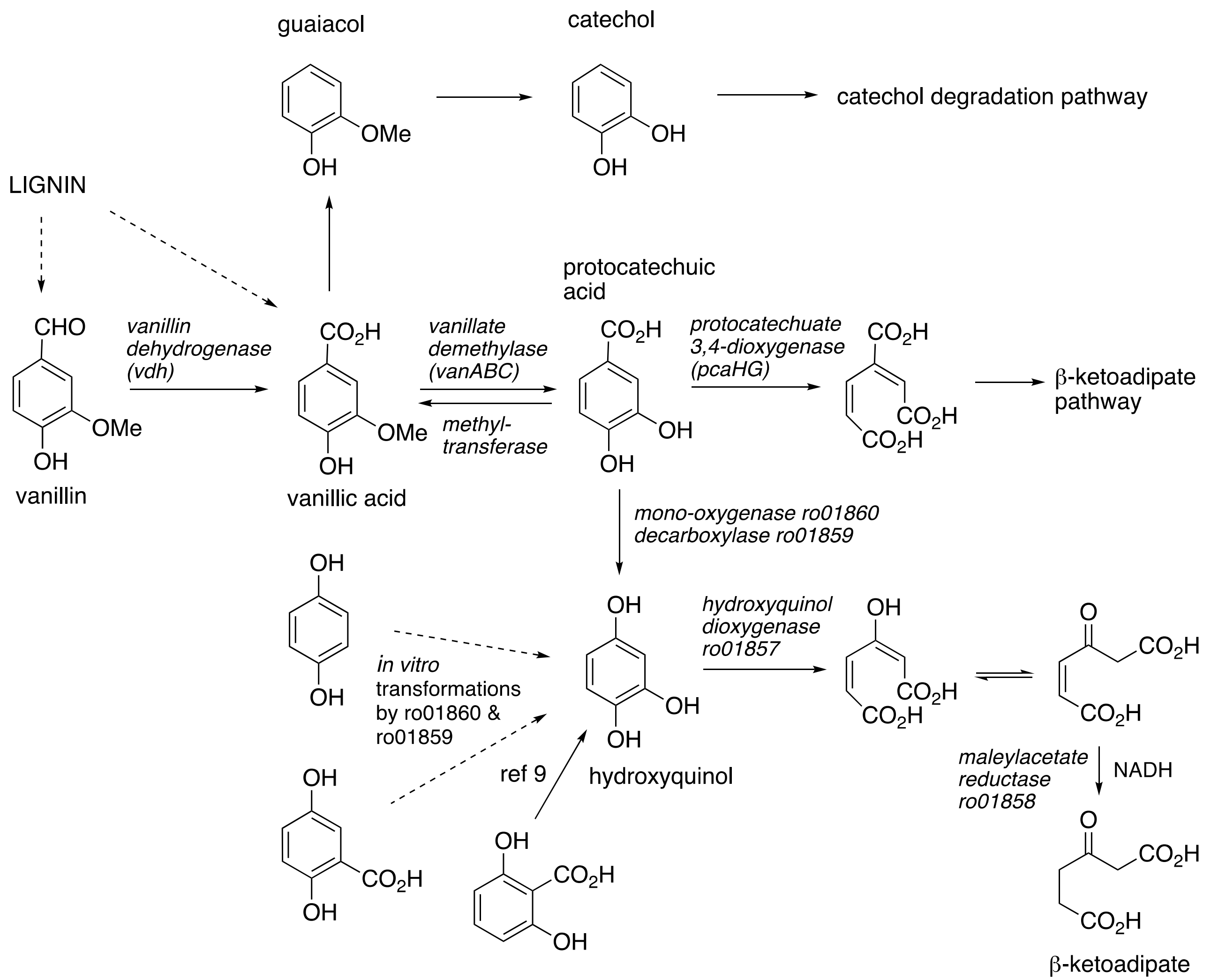

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3 Figure 6