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ORIGINAL PAPER

# Flavin-containing monooxygenases from *Phanerochaete chrysosporium* responsible for fungal metabolism of phenolic compounds

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**Abstract** We investigated the cellular responses of the white-rot basidiomycete *Phanerochaete chrysosporium* against vanillin. Based upon a proteomic survey, it was demonstrated that two flavin-containing monooxygenases (PcFMO1 and PcFMO2) are translationally up-regulated in response to exogenous addition of vanillin. To elucidate their catalytic functions, we cloned cDNAs and heterologously expressed them in *Escherichia coli*. The recombinant PcFMO1 showed catalytic activities against monocyclic phenols such as phenol, hydroquinone, and 4-chlorophenol. In addition, the product from hydroquinone was identified as 1,2,4-trihydroxybenzene, an important intermediate in a metabolic pathway of aromatic compounds in which the aromatic ring of 1,2,4-trihydroxybenzene can be further cleaved by fungal dioxygenases for mineralization. Thus, the ortho-cleavage pathway of phenolic compounds would presumably be associated with PcFMO1.

**Keywords** White-rot basidiomycete · Proteomics · Flavin-containing monooxygenase · Phenol hydoxylase · Lignin degradation

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## Introduction

White-rot basidiomycetes are the only organisms known to be responsible for the complete mineralization of woody components including lignin, one of the most recalcitrant biomaterials on earth (Eriksson et al. 1990). White-rot basidiomycetes commonly share unique extracellular enzymes such as lignin, manganese peroxidases, and laccase. These enzymes facilitate decomposition of polymeric lignin via non-specific oxidation, resulting in the generation of a wide variety of aromatic fragments (Hammel and Moen 1991; Wariishi et al. 1991). Since the aromatic fragments including phenolic compounds are further metabolized and mineralized intracellularly, basidiomycetes should possess versatile intracellular metabolic systems. Recently, various "omics" studies have

been performed to increase systematic understanding of the metabolic diversity of basidiomycetes (Shimizu et al. 2005; Sato et al. 2009; Vanden Wymelenberg et al. 2009). We have extensively investigated cellular responses of *Phanerochaete chrysosporium* to lignin-related compounds (Shimizu et al. 2005; Matsuzaki et al. 2008; Nakamura et al. 2010), and demonstrated that in the white-rot basidiomycete *P. chrysosporium* a series of intracellular enzymes are induced by exogenous addition of vanillin. Since vanillin is an abundant and key intermediate in the lignin biodegradation process, cellular responses to vanillin would be compulsory to optimize ligninolytic conditions in fungal cells. It is of great interest therefore to increase understanding of the catalytic functions of vanillin-responsive enzymes.

Flavin-containing monooxygenases (FMOs) are widely distributed within living organisms, and are involved in various biological processes such as the detoxification of drugs, biodegradation of environmental aromatic compounds, and biosynthesis of antibiotics (Ballou et al. 2005; van Berkel et al. 2006; Hao et al. 2009). Although a number of genomic projects have highlighted the molecular divergence of FMOs within the fungal kingdom, their biochemical features including transcriptional/translational regulations and catalytic properties remain obscure. Among a number of FMO-dependent reactions, hydroxylation reactions of phenolic compounds to their corresponding *o*-diol derivatives, catalyzed by phenol hydroxylase (PH), are important for the biodegradation of various aromatic compounds (Ballou et al. 2005; Vaillancourt et al. 2006). Recently, it has also been demonstrated that the white-rot basidiomycete *Trametes versicolor* is capable of growing with phenol as its sole carbon source, and PH plays an important role in its catabolic system (Yemendzhiev et al. 2008; Alexieva et al. 2010).

Here we demonstrate that two FMOs from *P. chrysosporium* were significantly induced by an exogenous addition of vanillin. Using recombinant enzymes, it was clear that a FMO showed PH activity to catalyze *o*-hydroxylation of a series of phenolic compounds such as phenol and catechol. This is the first report describing molecular and functional characterization of a basidiomycetous FMO exhibiting PH activity. These results also suggest that vanillin may be responsible for activation of fungal metabolic pathways for phenolic compounds.

## Materials and methods

### Chemicals

1,2,4-Trihydroxybenzene (1,2,4-THB) and 4-chlorocatechol were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). All other chemicals used were purchased from Wako Pure Chemicals. Deionized water was obtained using a Milli-Q System (Millipore).

### Organism and culture conditions

*Phanerochaete chrysosporium* (ATCC 34541) was grown from conidial inocula at 37°C in a stationary culture under aerobic conditions (20 ml of medium in 200 ml Erlenmeyer flasks). The medium (pH 4.5) contained 0.5% D-glucose (high carbon) and 1.2 mM ammonium tartrate (low nitrogen) as carbon and nitrogen sources, as described previously (Shimizu et al. 2005). After a 2 day incubation, vanillin (4-hydroxy-3-methoxybenzaldehyde) in acetonitrile (80 μl) was added to make a final concentration of 2.0 mM. For the control culture, only acetonitrile (80 μl) was added.

### Proteomic analysis

The pellicle mat was incubated with vanillin for 48 h at 37°C. The mat was isolated from the medium by suction filtration, washed twice with sterilized water, dried with a paper towel, frozen under liquid nitrogen, and crushed into a fine powder using a mortar and pestle. Intracellular proteins were extracted using SDS buffer (4% SDS, 2% DTT, 20% glycerol, 20 mM PMSF, and 100 mM Tris–HCl) (Shimizu et al. 2005; Nakamura et al. 2010). After 3 h incubation at room temperature (20°C), cell debris were removed by centrifugation and the extracted proteins were precipitated by the addition of four volumes of cold acetone and incubated overnight at −20°C (O'Farrell 1975). The resultant protein pellet was solubilized in urea buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.12% DeStreak reagent (GE Healthcare), 0.5% IPG buffer (pH 3–10 NL, GE Healthcare), and a small amount of bromophenol blue.

Two-dimensional gel electrophoresis (2-DE), in-gel tryptic digestion, and MALDI-TOF–MS analysis were performed as described previously (Shimizu

et al. 2005; Nakamura et al. 2010). Identification of proteins by peptide mass fingerprinting (PMF) analysis was carried out using MASCOT search (Matrix Science) against a *P. chrysosporium* in silico protein database generated from JGI/DOE genomic annotation data (version 1.0 or 2.0). This was used in combination with our own annotation data.

### Isolation and cloning of cDNAs encoding PcFMOs

A pellicle mat of *P. chrysosporium* was cultivated with 2.0 mM vanillin for 48 h, and then total RNA was isolated using an RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The reverse transcription (RT) reaction was carried out using SuperScript III reverse transcriptase (Invitrogen), as previously described (Nakamura et al. 2010). The cDNA fragments of PcFMOs were amplified using Pyrobest DNA polymerase (TaKaRa) with the primer combinations FMO1-F/FMO1-R and FMO2-F/FMO2-R (Table S1). PCR conditions were as previously described (Nakamura et al. 2010). PCR products were then cloned into a pGEM vector (Promega) and sequenced using an Applied Biosystems 3130*xl* Genetic Analyzer. The nucleotide and deduced amino acid sequences were analyzed using BLAST and BLASTP search programs. Alignment of amino acid sequences was conducted using the ClustalW program available online (http://align.genome.jp/).

### Expression of recombinant protein and preparation of cell-free extracts

Expression plasmids of PcFMO1 and PcFMO2 were constructed using the pET-22 vector system (TaKaRa) and designed to produce a recombinant protein with a C-terminal histidine-tag. The cDNA for *E. coli* expression was amplified from the plasmid. The primer sequences for expression plasmid construction are summarized in Table S1. The PCR mixture (50 µl) contained 1 U Phusion High-Fidelity DNA Polymerase (NEB) in a 50 µl reaction mixture containing 1 × Phusion HF Buffer, 200 µM dNTPs, 1 µl plasmid template, and 100 pmol each of specific primers (FMO1-Fex, FMO1-Rex, FMO2-Fex, FMO2-Rex). PCR conditions were as follows: 94°C for 60 s followed by 94°C for 10 s, 60°C for 15 s, and 72°C for 60 s for 28 cycles, and then a final 3 min extension at 72°C. Amplified cDNA fragments were digested with selected endonucleases, and subcloned into a pET-22 vector that had been treated with the same endonucleases. The recombinant plasmids were transferred into *E. coli* BL21 (DE3) pLysS strain (TaKaRa). Transformants were grown in 1.0 l of TB broth containing ampicillin (10 µg/ml) with shaking (180 rpm) at 37°C until the $OD_{600}$ reached 0.5. The culture was induced by the addition of isopropyl-1-thio-$\beta$-D-galactopyranoside at a final concentration of 0.5 mM, and bacterial cells were incubated for another 24 h at 27°C. Bacterial pellets were harvested by centrifugation at 7,000*g* for 5 min at 4°C. The cells in a pellet were washed twice with 50 mM HEPES buffer (pH 7.4), and resuspended in 30 ml HEPES buffer. Cells were disrupted by freeze–thaw cycles and sonication, and the mixture was centrifuged at 15,000*g* for 20 min at 4°C. Resultant supernatants were used as cell-free extracts.

### Purification of recombinant protein

Cell-free extracts were applied to a His GraviTrap (GE Healthcare) column that had been equilibrated with binding buffer containing 20 mM imidazole. Unbound proteins were removed from the column by washing with binding buffer. The bound recombinant proteins were eluted from the column using elution buffer containing 500 mM imidazole. After purification, the elution buffer was replaced with 50 mM HEPES buffer (pH 7.5) using a PD-10 desalting column (GE Healthcare). The purified protein solutions were stored at −80°C for further analysis. Concentration of proteins was determined using a DC Protein Assay Kit (Bio-Rad). Purified enzymes were analyzed using 12% SDS-PAGE (Nakamura et al. 2010). UV–vis absorption spectra were recorded on a HITACHI U3000 spectrometer with a 1 cm quartz cuvette.

### Enzyme assays

Enzymatic activity was determined from the NADPH oxidation ratio by monitoring the change in absorbance at 340 nm ($\varepsilon 340 = 6.2 \times 10^3\ M^{-1}\ cm^{-1}$) using an HITACHI U3000 spectrometer. The reaction mixture (1 ml) consisted of recombinant protein (5 µg), substrates (100 µM), NADPH (200 µM), glycerol (20% w/v), and 50 mM HEPES buffer (pH 7.5). The reaction was initiated by the addition of 10 µL NADPH solution. Reaction products and corresponding

authentic standards were analyzed by high performance liquid chromatography (HPLC; Shimadzu LC-10AD system) using a Shimadzu STR ODS-II column with a linear gradient from 20% acetonitrile in water (0.05% phosphoric acid; isocratic for 5 min) to 100% acetonitrile (21–30 min) at a flow rate of 1.0 ml/min. A UV monitor was utilized at 220 or 230 nm. Products were identified by comparing their retention times on HPLC with authentic standards. The authentic standards were eluted with a retention time of 2.2 min for 1,2,4-THB, 2.6 min for pyrogallol, 2.9 min for hydroquinone, 4.6 min for catechol, 7.6 min for 3-methylcatechol, 8.8 min for 4-methylcatechol, 12.8 min for *m*-cresol, 12.9 min for 4-chlorocatechol, 13.6 min for 4-bromocatechol, 13.8 min for *p*-cresol, and 15.3 min for 4-chlorophenol.

## Results

### Proteomic differential display analysis

To elucidate cellular responses to exogenous addition of vanillin, proteomic differential display analysis of intracellular proteins was performed using 2-DE. Figure 1 depicts the expression profiles of intracellular proteins obtained from control and vanillin-treated cells of *P. chrysosporium*. Through the proteomic survey, it was clearly shown that several proteins were specifically expressed and/or significantly induced in the vanillin-treated cells (Fig. 1). Using PMF analysis, two proteins that appeared in the vanillin-treated cells were identified as FMOs, and designated PcFMO1 and PcFMO2 (Fig. 1). The migration distances of PcFMO1 and PcFMO2 most closely agreed with the theoretical p*I* and molecular mass deduced from primary sequences (Table S2).

### Isolation and characterization of cDNA of PcFMOs

To better understand the genetic features of PcFMOs, cDNAs with open reading frames (ORFs) were cloned and sequenced. The cDNA of PcFMO1 encoded an ORF consisting of 1,815 bp nucleotides encoding 604 amino acids, in which ten introns were spliced (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). The ORF of PcFMO2 had 1,746 bp nucleotides and encoded 581 amino acids, in which eight introns were spliced. The cDNAs of PcFMO1 and PCFMO2 had 23

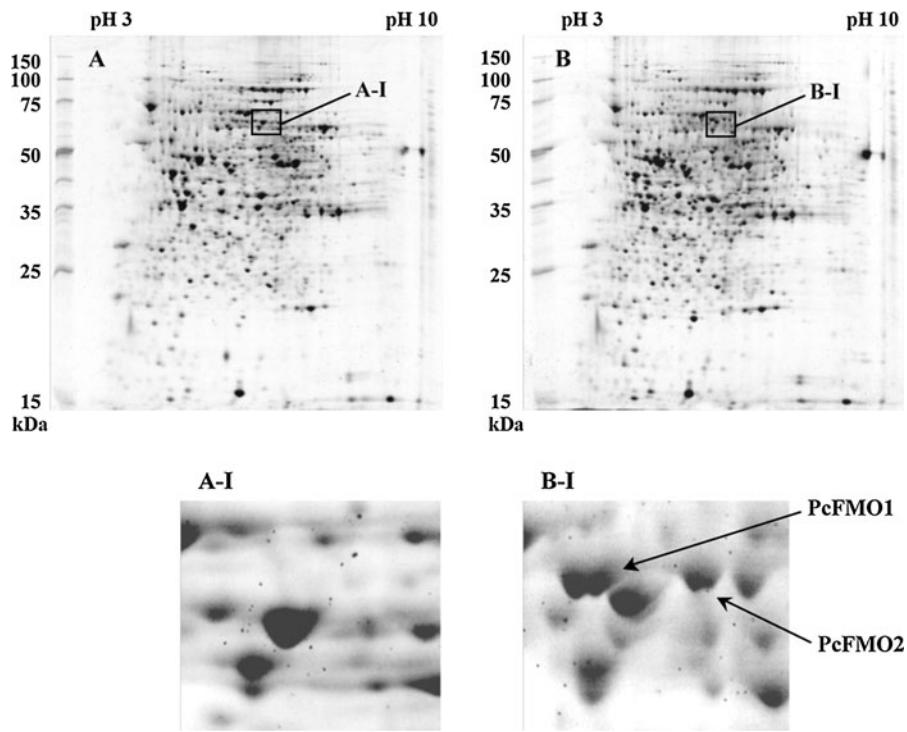


**Fig. 1** Proteome map of intracellular proteins extracted from *P. chrysosporium*. Intracellular proteins without (**a**) or with (**b**) vanillin 48 h after a 2 day preincubation. Proteins were extracted from the mycelial mat and expanded 2-dimensionally by isoelectric focusing (*horizontal*) and SDS–polyacrylamide gel electrophoresis (*vertical*)

and 34 single-nucleotide substitutions. Since these substitutions were also found in the genomic DNA, the mutations would presumably reflect polymorphisms in *P. chrysosporium*. The deduced amino acid sequences of PcFMO1 and PcFMO2 showed 21.2% identity and 40.2% similarity (Fig. S1). As shown in Fig. 2, PcFMO1 and PcFMO2 conserved the protein architecture of the FMO such that (i) the flavin adenine dinucleotide (FAD)-binding region was found in the sequence, (ii) a common motif of the FAD/NAD(P)H-dependent oxidoreductase (GxGxxG sequence for $\beta\alpha\beta$-fold fingerprint) was found in the flanking regions of the N-terminal, and (iii) a highly conserved GD sequence that interacts with the ribose moiety of the FAD was found at the center of the polypeptide (Krueger and Williams. 2005; Schlaich 2007). Based upon overall protein sequence similarity, PcFMO1 showed a significant similarity to various FAD-dependent monooxygenases, and the highest similarity (58% similarity and 44% identity) to a PH from the soil yeast *Trichosporon cutaneum* (Kälin et al. 1992). However, sequence similarity between PcFMO2 and *T. cutaneum* PH was lower with 46% similarity and 31% identity.

## Phenol hydroxylase activities of PcFMOs

Using the isolated cDNA, PcFMO1, and PcFMO2 were heterologously expressed in *E. coli* and purified by histidine-tag affinity column chromatography (Fig. 3a). The purified PcFMOs were visibly yellow in solution, indicative of a bound flavin prosthetic group. As shown in Fig. 3b, the absorption spectrum of PcFMO1 was similar to that of other FMOs with absorption peaks at 372 and 443 nm and a shoulder at 465 nm. In contrast, a broad absorption spectrum with

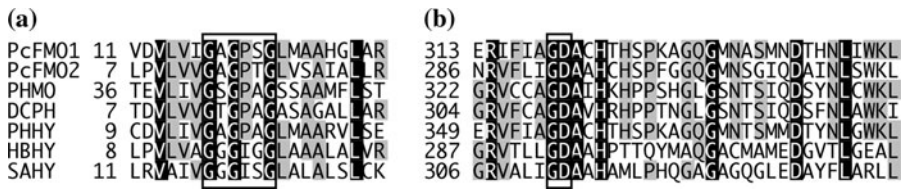


**Fig. 2** Alignment analysis of the putative two FAD-binding regions in PH and other aromatic hydroxylases. **a** First and **b** second FAD-binding regions. Conserved "GXGXXG" and "GD" motif sequences are shown in *boxes*. Abbreviations: *PcFMO1 and PcFMO2*, FMOs from *P. chrysosporium* (this study); *PHHY*, phenol hydroxylase from *T. cutaneum* (Kälin et al. 1992); *PHMO*, phenol monooxygenase from *Pseudomonas* sp. (Nurk et al. 1991); *DCPH*, 2,4-dichlorophenol hydroxylase from *Alcaligenes eutrophus* (Perkins et al. 1990); *HBHY*, 3-hydroxybenzoate 6-hydroxylase from *Polaromonas naphthalenivorans* (Park et al. 2007); *SAHY*, salicylate hydroxylase from *Pseudomonas putida* (Lee et al. 1996)

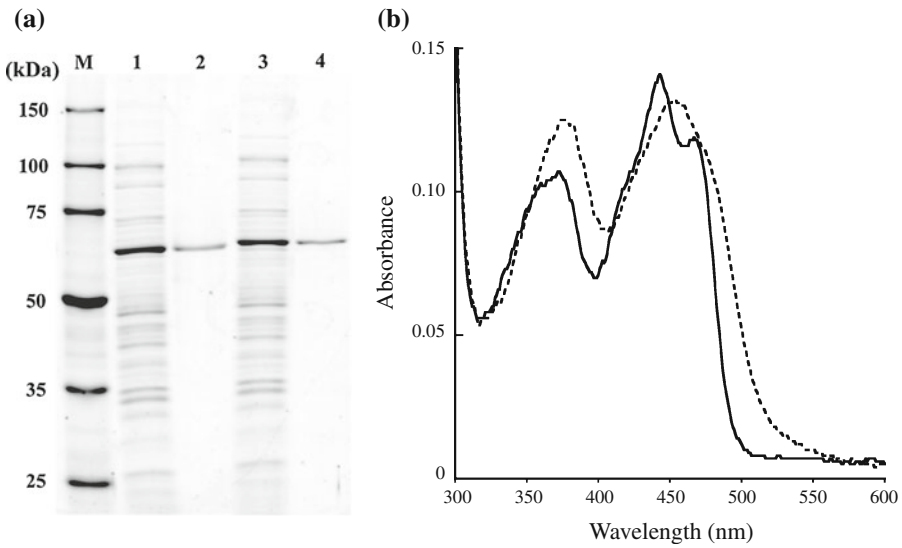


**Fig. 3** Heterologous expression and spectroscopic analysis of PcFMOs. **a** SDS-PAGE analysis of purified recombinant enzyme. *Lane M*, molecular weight marker; *Lane 1*, crude cell extract containing PcFMO1; *Lane 2*, purified PcFMO1; *Lane 3*, crude cell extract containing PcFMO2; *Lane 4*, purified PcFMO2. **b** UV–vis absorption spectra of purified PcFMO1 (*solid line*) and PcFMO2 (*broken line*)

absorption peaks at 380 and 450 nm was observed from PcFMO2. To evaluate catalytic activity, phenol hydroxylation reactions were performed using a series of phenolic compounds including vanillin. Neither PcFMO1 nor PcFMO2 exhibited catalytic activities against vanillin, vanillyl alcohol, or vanillic acid. When phenol was used as a substrate, PcFMO1 effectively catalyzed the hydroxylation reaction under pH conditions between 5.0 and 9.5; maximum activity was observed at around pH 7.5 (data not shown). The catalytic conversion of phenol by PcFMO1 was strictly dependent on NADPH as an electron donor. However, no catalytic conversion of phenolic compounds was observed for PcFMO2. We then intensively investigated the catalytic properties of PcFMO1 using a wide range of phenolic compounds. PcFMO1 was shown to be capable of converting a series of monocyclic substrates, including phenol, catechol, hydroquinone, *m*-cresol, *p*-cresol, 4-bromophenol, and 4-chlorophenol (Table 1). The formation of *o*-diol compounds was shown by comparison of their HPLC retention times with authentic standards. However, ortho-substituted phenolic compounds such as *o*-cresol, 2-methoxyphenol, and methoxyhydroquinone were not converted by PcFMO1 (Table 1).

## Discussion

A proteomic differential display analysis revealed the significant up-regulation of PcFMO1 and PcFMO2 in response to the exogenous addition of vanillin. Because vanillin is one of the most important key intermediates during the lignin biodegradation process by fungi, cellular responses to vanillin would be compulsory in order to reorganize the intracellular environment into ligninolytic condition (Shimizu et al. 2005; Nakamura et al. 2010). Although PcFMO1 and PcFMO2 did not exhibit substantial activity against vanillin itself, their translational up-regulation may suggest some contribution to lignin degradation processes by activation of the metabolic pathways of phenolic compounds.

To elucidate the catalytic activities of PcFMOs, recombinant enzymes were expressed using an *E. coli* expression system. Recombinant PcFMO1 and PcFMO2 were expressed in the cytosolic fraction of *E. coli* and purified by histidine-tag affinity column chromatography (Fig. 3a). PcFMO1 had a similar absorption spectrum to the PH from *T. cutaneum* and to hydroquinone hydroxylase from *Candida parapsilosis* (Eppink et al. 2000). Substantial activities against

**Table 1** Activities of recombinant PcFMO1 toward phenol analogs

| Substrate | Activity (μmol/min/mg protein)[a] | Relative activity (%)[b] | Estimated reaction product |
|---|---|---|---|
| Phenol | 16.8 | 100 | Catechol, Pyrogallol |
| Catechol | 5.2 | 31 | Pyrogallol |
| Hydroquinone[c] | 7.1 | 42 | 1,2,4-Trihydroxybenzene |
| *m*-Cresol | 2.9 | 17 | 3-Methylcatechol |
| *p*-Cresol | 4.2 | 25 | 4-Methylcatechol |
| 4-Bromophenol | 8.7 | 52 | 4-Bromocatechol |
| 4-Chlorophenol | 10.3 | 61 | 4-Chlorocatechol |
| *o*-Cresol | – | – | Not detected |
| 2-Methoxyphenol | – | – | Not detected |
| 4-Nitrophenol | – | – | Not detected |
| Methoxyhydroquinone | – | – | Not detected |
| 1-Naphtol | – | – | Not detected |
| Pyrogallol | – | – | Not detected |
| 1,2,4-Trihydroxybenzene | – | – | Not detected |

[a] Activities were calculated using molar absorbance coefficient of NADPH ($\varepsilon 340 = 6.2 \times 10^3\ M^{-1}\ cm^{-1}$)

[b] Relative activities are given as percentages of the activity toward phenol

[c] The reaction mixture contained 10 μM dithiothreitol to prevent self-oxidation of hydroquinone

various phenolic compounds implied that recombinant PcFMO1 was successfully expressed as an active form. In contrast, PcFMO2 showed broad absorption spectra similar to that of FAD in the buffer. In addition, no catalytic conversion of prototypic substrates by PcFMO2 was observed. Although further investigation should be directed at the spectroscopic and functional characterization of PcFMO2, it is possible that recombinant PcFMO2 was expressed with incorrect protein folding, as a catalytically inactive form.

A series of monocyclic phenolic compounds were converted by PcFMO1; nevertheless, phenolic compounds with an ortho substituent such as *o*-cresol, 2-methoxyphenol and methoxyhydroquinone were not converted. This suggests possible mechanisms by which the enzyme recognizes the ortho position of phenolic substrates. Moreover, the chemical properties of the para substituent seem to affect reaction efficiency; in fact, PcFMO1 showed significant catalytic activity against 4-chloropheonol, 4-bromophonol, and *p*-cresol but not against 4-nitrophenol (Table 1). Its catalytic properties were similar to those of PH from the basidiomycetous yeast *T. cutaneum* (Kälin et al. 1992) even though they share only 44% sequence identity. A single-component PH has been identified from the soil bacterium *Pseudomonas pickettii* (Kukor and Olsen 1992); although sequence similarity between PcFMO1 and the bacterial PH is only 20%. It has been reported that PH from *P. pickettii* is capable of converting *o*-, *m*-, and *p*-cresol with 81, 110, and 102% relative activity toward phenol. However, PcFMO1 converted *m*-, and *p*-cresol with 17 and 25% relative activity toward phenol, and no activity against *o*-cresol. In contrast, PcFMO1 converted 4-chlorophenol with 61% relative activity toward phenol, but PH from *P. pickettii* exhibited 20% relative activity against this compound. These marked differences in substrate specificity are suggestive of different evolutional strategies involved in molecular and functional diversification of bacterial and fungal PH.

Based on metabolic studies, it has been reported that a white-rot basidiomycete, *T. versicolor*, is capable of degrading phenol intracellularly and utilizing it as a carbon source (Yemendzhiev et al. 2008; Alexieva et al. 2010). It is likely that the catabolic process involves a phenol hydroxylation reaction to produce catechol and/or 1,2,4-THB; key intermediates for energy production via the ortho-cleavage pathway (Reddy et al. 1998; Reddy and Gold 2000; Kitagawa et al. 2004). We demonstrated the catalytic activity of recombinant PcFMO1 in the production of catechol and 1,2,4-THB from phenol. To the best of our knowledge, this is the first report showing a phenol hydroxylation reaction in vitro by a recombinant enzyme of *P. chrysosporium*. The enzyme catalyzing the ring fission reaction of 1,2,4-THB has also been isolated and characterized from *P. chrysosporium* (Rieble et al. 1994). The cleavage of the phenol ring is thought to be one of the most important steps in the mineralization of many kinds of aromatic compounds including lignin and organopollutants (Vaillancourt et al. 2006; Lipscomb 2008), suggesting the biological importance of PcFMO1.

In conclusion, we revealed up-regulation of PcFMO1 and PcFMO2 in *P. chrysosporium* responding to exogenous addition of vanillin. PcFMO1 showed PH activity to catalyze *o*-hydroxylation of a series of phenolic compounds such as phenol and catechol, although it showed no activity against vanillin itself. Our results suggest that PcFMO1 plays important roles in the catabolic mineralization of phenolic compounds. Moreover, it is possible that vanillin has a role in the activation of fungal metabolic pathways for the biodegradation of phenolic compounds.

## References

Alexieva Z, Yemendzhiev H, Zlateva P (2010) Cresols utilization by *Trametes versicolor* and substrate interactions in the mixture with phenol. Biodegradation 21:625–635

Ballou DP, Entsch B, Cole LJ (2005) Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases. Biochem Biophys Res Commun 338:590–598

Eppink MH, Cammaart E, Van Wassenaar D, Middelhoven WJ, van Berkel WJ (2000) Purification and properties of hydroquinone hydroxylase, a FAD-dependent monooxygenase involved in the catabolism of 4-hydroxybenzoate in *Candida parapsilosis* CBS604. Eur J Biochem 267:6832–6840

Eriksson KEL, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood and wood components. Springer, Berlin

Hammel KE, Moen MA (1991) Depolymerization of a synthetic lignin in vitro by lignin peroxidase. Enzym Microb Technol 13:15–18

Hao D, Chen S, Mu J, Xiao P (2009) Molecular phylogeny, long-term evolution, and functional divergence of flavin-containing monooxygenases. Genetica 137:173–187

Kälin M, Neujahr HY, Weissmahr RN, Sejlitz T, Jöhl R, Fiechter A, Reiser J (1992) Phenol hydroxylase from *Trichosporon cutaneum*: gene cloning, sequence analysis, and functional expression in *Escherichia coli*. J Bacteriol 174:7112–7120

Kitagawa W, Kimura N, Kamagata Y (2004) A novel *p*-nitrophenol degradation gene cluster from a gram-positive bacterium, *Rhodococcus opacus* SAO101. J Bacteriol 186: 4894–4902

Krueger SK, Williams DE (2005) Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol Theor 106: 357–387

Kukor JJ, Olsen RH (1992) Complete nucleotide sequence of tbuD, the gene encoding phenol/cresol hydroxylase from *Pseudomonas pickettii* PKO1, and functional analysis of the encoded enzyme. J Bacteriol 174:6518–6526

Lee J, Oh J, Min KR, Kim Y (1996) Nucleotide sequence of salicylate hydroxylase gene and its 5′-flanking region of *Pseudomonas putida* KF715. Biochem Biophys Res Commun 17:544–548

Lipscomb JD (2008) Mechanism of extradiol aromatic ring-cleaving dioxygenases. Curr Opin Struct Biol 18:644–649

Matsuzaki F, Shimizu M, Wariishi H (2008) Proteomic and metabolomic analyses of the white-rot fungus *Phanerochaete chrysosporium* exposed to exogenous benzoic acid. J Proteome Res 7:2342–2350

Nakamura T, Ichinose H, Wariishi H (2010) Cloning and heterologous expression of two aryl-aldehyde dehydrogenases from the white-rot basidiomycete *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 394:470–475

Nurk A, Kasak L, Kivisaar M (1991) Sequence of the gene (pheA) encoding phenol monooxygenase from Pseudomonas sp. EST1001: expression in *Escherichia coli* and *Pseudomonas putida*. Gene 15:13–18

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021

Park M, Jeon Y, Jang HH, Ro HS, Park W, Madsen EL, Jeon CO (2007) Molecular and biochemical characterization of 3-hydroxybenzoate 6-hydroxylase from *Polaromonas naphthalenivorans* CJ2. Appl Environ Microbiol 73(16): 5146–5152

Perkins EJ, Gordon MP, Caceres O, Lurquin PF (1990) Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J Bacteriol 172:2351–2359

Reddy GV, Gold MH (2000) Degradation of pentachlorophenol by *Phanerochaete chrysosporium*: intermediates and reactions involved. Microbiology 146:405–413

Reddy GV, Gelpke MD, Gold MH (1998) Degradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium*: involvement of reductive dechlorination. J Bacteriol 180: 5159–5164

Rieble S, Joshi DK, Gold MH (1994) Purification and characterization of a 1,2,4-trihydroxybenzene 1,2-dioxygenase from the basidiomycete *Phanerochaete chrysosporium*. J Bacteriol 176:4838–4844

Sato S, Feltus FA, Iyer P, Tien M (2009) The first genome-level transcriptome of the wood-degrading fungus *Phanerochaete chrysosporium* grown on red oak. Curr Genet 55:273–286

Schlaich NL (2007) Flavin-containing monooxygenases in plants: looking beyond detox. Trends Plant Sci 12:412–418

Shimizu M, Yuda N, Nakamura T, Tanaka H, Wariishi H (2005) Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. Proteomics 5:3919–3931

Vaillancourt FH, Bolin JT, Eltis LD (2006) The ins and outs of ring-cleaving dioxygenases. Crit Rev Biochem Mol Biol 41:241–267

van Berkel WJ, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J Biotechnol 124:670–689

Vanden Wymelenberg A, Gaskell J, Mozuch M, Kersten P, Sabat G, Martinez D, Cullen D (2009) Transcriptome and secretome analyses of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. Appl Environ Microbiol 75:4058–4068

Wariishi H, Valli K, Gold MH (1991) In vitro depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 176: 269–275

Yemendzhiev H, Gerginova M, Krastanov A, Stoilova I, Alexieva Z (2008) Growth of *Trametes versicolor* on phenol. J Ind Microbiol Biotechnol 35:1309–1312