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Cloning and heterologous expression of two aryl-aldehyde dehydrogenases from the white-rot basidiomycete *Phanerochaete chrysosporium*

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ABSTRACT

We identified two aryl-aldehyde dehydrogenase proteins (PcALDH1 and PcALDH2) from the white-rot basidiomycete *Phanerochaete chrysosporium*. Both PcALDHs were translationally up-regulated in response to exogenous addition of vanillin, one of the key aromatic compounds in the pathway of lignin degradation by basidiomycetes. To clarify the catalytic functions of PcALDHs, we isolated full-length cDNAs encoding these proteins and heterologously expressed the recombinant enzymes using a pET/*Escherichia coli* system. The open reading frames of both PcALDH1 and PcALDH2 consisted of 1503 nucleotides. The deduced amino acid sequences of both proteins showed high homologies with aryl-aldehyde dehydrogenases from other organisms and contained ten conserved domains of ALDHs. Moreover, a novel glycine-rich motif "GxGxxxG" was located at the NAD^+-binding site. The recombinant PcALDHs catalyzed dehydrogenation reactions of several aryl-aldehyde compounds, including vanillin, to their corresponding aromatic acids. These results strongly suggested that PcALDHs metabolize aryl-aldehyde compounds generated during fungal degradation of lignin and various aromatic xenobiotics.

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Introduction

White-rot basidiomycetes are the only known organisms that completely mineralize woody components, including lignin – one of the most recalcitrant biomaterials on Earth [1–5]. White-rot basidiomycetes commonly share unique extracellular enzymes such as lignin and manganese peroxidases (LiP and MnP, respectively) and laccase (Lac) [2,6]. These enzymes trigger decomposition of polymeric lignin via non-specific oxidation, resulting in the formation of a wide variety of aromatic fragments [7,8]. Such fragments are further metabolized and finally mineralized intracellularly [2,4,7,8]. Extracellular ligninolytic enzymes have been extensively investigated and biochemically characterized. However, the intracellular metabolic systems involved in the lignin degradation process remain unclear. The whole-genome sequence of the white-rot basidiomycete *Phanerochaete chrysosporium* has recently become available to the public (http://www.jgi.doe.gov/). As reported by JGI/DOE, the 35.1 Mb euchromatic genome of this fungus contains 10,048 genes [9]. Many researchers have taken advantage of this genomic data, and have carried out various "Omics" studies to increase understanding of ligninolytic activities of basidiomycetes. Consequently, it has become clear that *P. chrysosporium* exhibits sophisticated cellular mechanisms in response to exogenous aromatic compounds, and that these mechanisms are regulated transcriptionally and translationally [10–12]. Therefore, systematic understanding of the regulation of genes, enzymes, and proteins, and their physiological functions, would be of great interest. However, the vast majority of gene products have not been functionally characterized.

Numerous aldehydes are generated from a series of bioprocesses in living organisms. Many aldehydes are toxic as a result of the electrophilic and reactive moiety of their carbonyl group. Therefore, organisms must have effective metabolic responses to detoxify these compounds to protect cellular components [13]. Aryl-aldehyde dehydrogenases (ALDHs) are widely distributed among living organisms and are thought to be important for metabolic detoxification of aromatic aldehydes [14]. Data from proteomic surveys suggest that expressions of several enzymes such as aryl-aldehyde dehydrogenases, aryl-alcohol dehydrogenase, and 1,4-benzoquinone reductase in *P. chrysosporium* are up-regulated by exogenous addition of vanillin; however, this compound detrimentally affects fungal growth [11]. Since vanillin is an abundant and key intermediate in the lignin biodegradation process, basidiomycetes presumably respond to this compound to activate and optimize ligninolytic processes. Thus, it is important to elucidate catalytic and physiological functions of vanillin-responsive

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enzymes. In the present study, we focused on two vanillin-responsive ALDHs, PcALDH1 and PcALDH2, from *P. chrysosporium*. We investigated their catalytic properties using heterologously expressed recombinant enzymes. The recombinant ALDHs showed catalytic activity against several aryl-aldehydes including vanillin, suggesting that PcALDH1 and PcALDH2 have key roles in lignin degradation. To the best of our knowledge, this is the first report on functional characterization of ALDHs from basidiomycetes.

Materials and methods

Chemicals. All chemicals were of analytical grade and were purchased from Wako Pure Chemicals.

Organism and culture conditions. P. chrysosporium (ATCC 34541) was grown from hyphae-inocula at 37 °C in a stationary culture (20 mL medium) under air. The medium (pH 4.5) used in this study contained 28 mM D-glucose and 1.2 mM ammonium tartrate (low nitrogen; LN) as the carbon and nitrogen sources, respectively, as described previously [11,15]. After a 2-day preincubation, vanillin (4-hydroxy-3-methoxybenzaldehyde) in acetonitrile (80 μL) was added to a final concentration of 2 mM. For the control culture, only acetonitrile (80 μL) was added.

Proteomic analysis. The mycelial mat was incubated with vanillin for 24 h at 37 °C. The mat was separated from the medium by filtration, washed with chilled water, frozen under liquid nitrogen, and ground 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) [16]. After 2 h incubation at room temperature, the extracted proteins were precipitated by addition of four volumes of cold acetone (−20 °C). The resultant 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), and a trace amount of bromophenol blue.

Two-dimensional gel electrophoresis (2-DE), in-gel tryptic digestion, and MALDI-TOF-MS analysis were performed as described previously [11]. Identification of proteins by peptide mass fingerprinting analysis was carried out using MASCOT search (Matrix Science) against a *P. chrysosporium in silico* protein database generated from genomic annotation data, which was used in combination with our own annotation data.

Isolation and cloning of cDNA encoding PcALDH1 and PcALDH2. A mycelial mat of *P. chrysosporium* was incubated with 2 mM vanillin for 1 day, and then total RNA was isolated using an RNeasy Plant Mini Kit (QIAGEN). The reverse transcription reaction (total volume, 20 μL) contained 200 U SuperScript III reverse transcriptase (Invitrogen) in 1× RT buffer containing 10 mM dithiothreitol, 0.5 mM dNTPs, 0.5 μg total RNA, and 10 pmol oligo$(dT)_{18}$ primer. The reaction mixture was incubated at 37 °C for 30 min, and then at 42 °C for 60 min. Target cDNA fragments were amplified with 1 U Phusion High-Fidelity DNA Polymerase (NEB) in a 50 μL reaction mixture containing 1× Phusion HF Buffer, 200 μM dNTPs, 2 μL template cDNA, and 100 pmol each of the upstream and downstream primers. Primer sequences are shown in Table S1. The PCR conditions were as follows: 98 °C for 30 s followed by 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 60 s for 30 cycles, and then a final 3-min extension at 72 °C. The PCR products were separated on a 1.5% agarose gel, visualized with a UV transilluminator, and purified using QIAquick PCR Purification Kit (QIAGEN). After purification, PCR products were treated with 1 U Ex Taq (TaKaRa) in 1× Ex Taq buffer and 200 μM dNTPs at 72 °C for 30 min, cloned into a pGEM-T Easy vector (Promega), and transformed into competent cells of *Escherichia coli* strain JM109. Positive clones were selected by blue/white screening and colony-direct PCR. Plasmids were isolated from 10 mL LB broth using a QIAprep Spin Miniprep Kit (QIAGEN) and sequenced with a CEQTM 2000XL DNA sequencing system (Beckman Coulter) after the sequencing reaction using the GenomeLab DTCS Quick Start Kit (Beckman Coulter). The nucleotide and deduced amino acid sequences were analyzed using BLAST and BLASTP search programs.

Expression of recombinant protein and preparation of cell-free extracts. Expression vectors were constructed using the pET-22 expression system. The coding regions for ALDHs were amplified from plasmid DNAs. cDNA fragments were amplified using specific primers that included restriction enzyme-recognition sequences (NdeI, upstream primer; HindIII, downstream primer). The PCR mixture (50 μL) contained 0.1 μg plasmid DNA, 2.5 U Pyrobest DNA polymerase (TaKaRa), 1× Pyrobest buffer II, 100 pmol each of the upstream and downstream primers, and dNTPs at a final concentration of 200 μM. The PCR conditions were as follows: 94 °C for 3 min followed by 94 °C for 20 s, 63 °C for 30 s, and 72 °C for 2 min for 28 cycles, and then a final 3-min extension at 72 °C. The amplified cDNA fragments were digested with NdeI and HindIII, and ligated into the pET-22 vector that had been treated with the same endonucleases. The recombinant plasmids containing the ALDH coding regions were transferred to into *E. coli* BL21 (DE3) pLysS (TaKaRa). The transformants were grown in TB broth containing ampicillin (10 μg/mL) with shaking (180 rpm) at 37 °C until the OD_{600} reached 0.5. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to the culture at a final concentration of 0.5 mM, and bacterial cells were incubated for 24 h with shaking (180 rpm) at 27 °C. Cell-free extracts were prepared as follows: bacterial cells were centrifuged at 7000*g* for 5 min at 4 °C. The cells in the pellet were washed 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. The supernatants were used as cell-free extracts.

Purification of recombinant protein. Recombinant proteins were purified from cell-free extracts using a histidine-tag (His-tag)-based purification procedure. Extracts were applied to a His GraviTrap (GE Healthcare) column that had been equilibrated with binding buffer containing 20 mM imidazole. Recombinant proteins containing a six-repeat histidine sequence at the C-terminal bound to the column. 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. Eluted recombinant proteins were separated by electrophoresis on a 12.5% SDS–polyacrylamide gel and visualized with SYPRO Red protein gel stain (TaKaRa). After purification, the elution buffer was replaced with storage buffer using a PD-10 desalting column (GE Healthcare). The purified protein solutions were stored at −80 °C until further analyses.

Enzyme assay. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad), which is based on the Lowry method. Enzymatic activity of recombinant ALDH (rALDH) was measured in a 1 mL reaction mixture consisting of recombinant protein (100 μg), substrates (100 μM), $tNAD^+$ (200 μM), glycerol (20% w/v), and 25 mM glycine–NaOH buffer (pH 10.0). The reaction was initiated by the addition of 10 μL $tNAD^+$ solution. The rALDH activity was determined as the initial tNADH production rates by monitoring the formation of tNADH at 400 nm using a UV–vis spectrophotometer.

Results and discussion

Proteomic differential display analysis

Expression profiles of cytosolic proteins were analyzed using a proteomic differential display technique. As shown in Fig. 1,

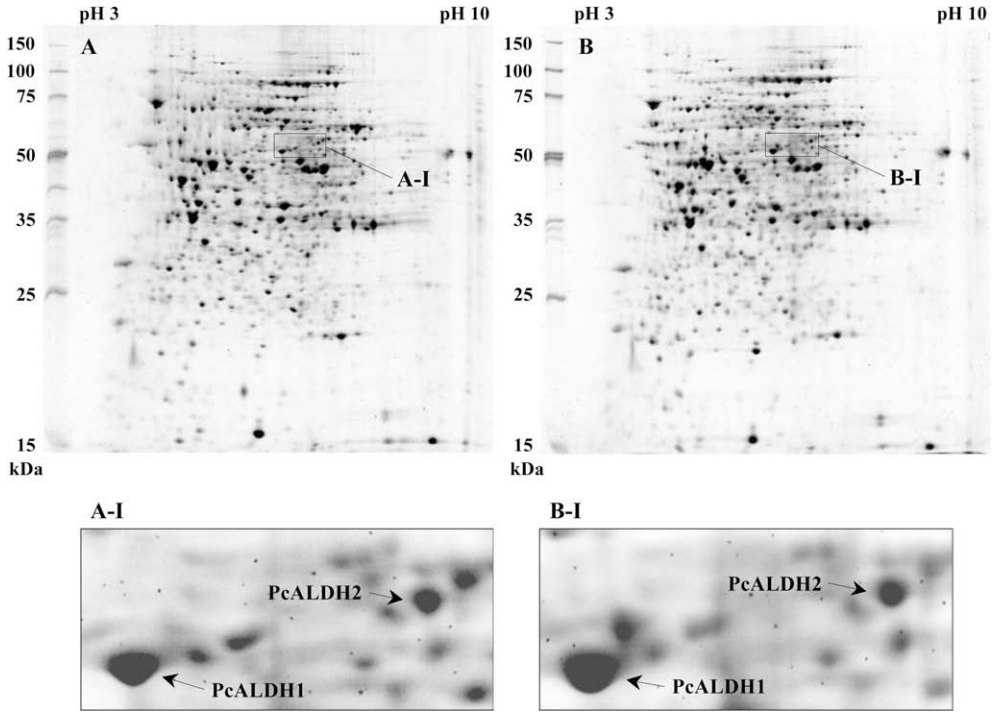

Fig. 1. Proteome map of intracellular proteins of *P. chrysosporium* grown without (A) or with (B) vanillin for 24 h after 2-day preincubation.

several intracellular proteins were up-regulated in vanillin-treated *P. chrysosporium*. Among several vanillin-responsive proteins, we identified two protein spots as PcALDH1 and PcALDH2 by PMF analysis (Table 1). The migration patterns of these two protein spots significantly matched the theoretical p*I* and molecular mass values of PcALDH1 and PcALDH2. The induction efficiency of PcALDH1 was slightly higher than that of PcALDH2. Moreover, PcALDH1 was more abundantly expressed than PcALDH2 in *P. chrysosporium* (Fig. 1). Therefore, PcALDH1 was assumed to have a more significant role in vanillin metabolism. A previous study showed that PcALDH1 and PcALDH2 were overexpressed when *P. chrysosporium* was treated with vanillin for 72 h [11]. However, fungal growth of *P. chrysosporium* was inhibited by exogenous addition of vanillin, with marked inhibitory effects visible within 24 h [11]. This result suggested that cellular responses to vanillin were very rapid. In fact, induction of PcALDHs occurred within 24 h after exposure to vanillin (Fig. 1). A rapid cellular response to induce PcALDH1 and PcALDH2 would be important for fungal metabolism of various aromatic aldehydes. The timing and coordination of such cellular response mechanisms should be determined using dynamic transcriptomic and proteomic profiling. Moreover, both PcALDH1 and PcALDH2 are responsive to benzoic acid [12], suggesting that PcALDH1 and PcALDH2 have important roles in metabolism of various aryl-aldehydes in fungi.

Table 1
P. chrysosporium ALDHs expressed in response to addition of vanillin.

Protein name	Putative function	Protein ID[a]	Levels of expression[b]	tpI[c]	tMW[d]	Cov.[e]
PcALDH1	Aryl-aldehyde dehydrogenase	133924	2.3	5.9	53.3	45
PcALDH2	Aryl-aldehyde dehydrogenase	137014	1.8	6.1	55.4	39

[a] Instant access number at JGI homepage (http://genomeportal.jgi-psf.org/Phchr1/Phchr1.home.html).
[b] Levels of expression in response to addition of vanillin.
[c] Theoretical p*I*.
[d] Theoretical mass.
[e] Sequence coverage (%) in peptide mass fingerprinting.

Isolation and characterization of cDNAs encoding PcALDHs

In the genomic database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html), putative coding nucleotide sequences of

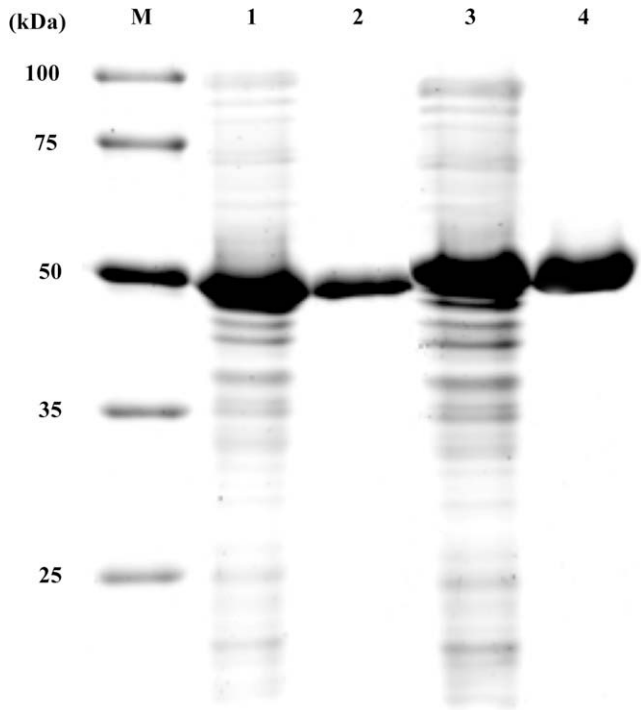

Fig. 2. Expression and purification of rPcALDHs. Lanes 1 and 3, crude cell extract containing rPcALDH1 and rPcALDH2, respectively; 2, purified rPcALDH1; 4, purified rPcALDH2.

PcALDH1 and PcALDH2 were predicted to have five and six introns, respectively. To elucidate their genetic features, we amplified the cDNA of each gene. We used RT-PCR to amplify each gene using gene-specific primers designed from flanking regions of putative start and stop codons, and obtained full-length cDNAs of PcALDH1 and PcALDH2. Open reading frames (ORFs) of both PcALDH1 and PcALDH2 consisted of 1503 bp nucleotides encoding 500 amino acids. The molecular weights of PcALDH1 and PcALDH2 were estimated to be 53.3 and 54.4 kDa, respectively. The deduced amino acid sequences of PcALDH1 and PcALDH2 showed 57.6% identity and 73.6% similarity. The experimentally deduced amino acid sequences and introns/exon boundaries in PcALDH1 and PcALDH2 matched with bioinformatically predicted sequences. However, the isolated cDNA of PcALDH1 had 19 single nucleotide substitutions that of PcALDH2 had 10. All mutations in both cDNAs were silent mutations. The nucleotide substitutions presumably reflect polymorphisms in *P. chrysosporium* spp. The deduced amino acid sequences showed high homology to a series of known ALDH sequences, especially those of other basidiomycetous proteins (Fig. S1). In addition, PcALDH1 and PcALDH2 each contained 10 ALDH motif sequences in which Glu269 and Cys303 residues are putative active sites [17,18]. Furthermore, a glycine-rich motif "GxGxxxG" in the NAD^+-binding domain was encoded between motifs 3 and 4 in both sequences [19,20].

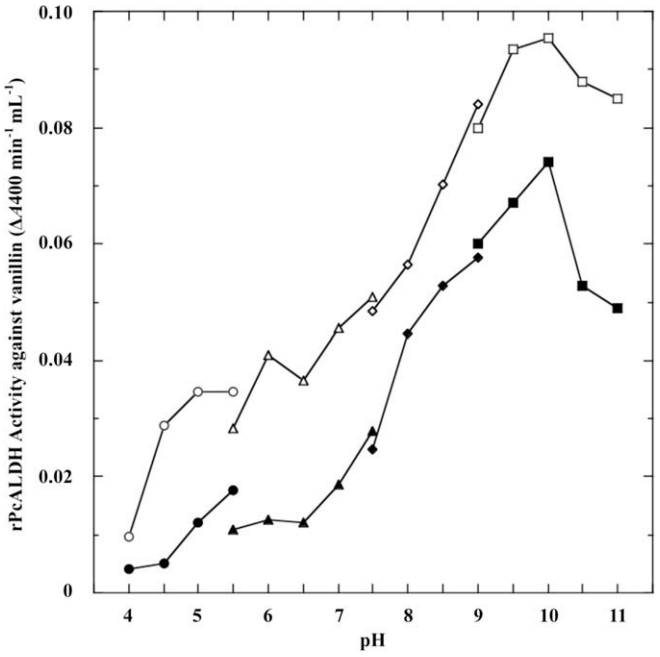

Fig. 3. pH-Dependency of rPcALDHs against vanillin. Reaction mixture (1 mL) consisted of $tNAD^+$ (100 μM), glycerol (20% w/v), and rPcALDHs (100 μg/mL). Buffers were as follows: 25 mM acetate buffer (circles), 25 mM phosphate buffer (triangles), 50 mM Tris–HCl buffer (diamonds), 25 mM glycine–NaOH buffer (squares). Open symbols represent rPcALDH1, closed symbols represent rPcALDH2.

Catalytic properties of recombinant PcALDH1 and PcALDH2

Recombinant PcALDH1 and PcALDH2 (rPcALDH1 and rPcALDH2) were expressed as C-terminal histidine-tagged proteins in *E. coli* cells and purified with a His-tag affinity column. The two rPcALDHs exhibited similar migration distances, and their molecular mass values matched those estimated from deduced amino acid sequences (Fig. 2). The enzymatic activities of purified rPcALDH1 and rPcALDH2 were determined using an assay in which vanillin was the substrate and $tNAD^+$ was coupled as an NAD^+ analogue. The use of $tNAD^+$ enabled detection of ALDH-mediated oxidation of $tNAD^+$ to tNADH by the change in absorbance at 400 nm without spectral interference caused by oxidation of aromatic aldehydes. The catalytic activities of rPcALDHs were pH-dependent, with maximum activities at around pH 10.0 (Fig. 3). Enzymatic reactions carried out at pH 4.0 showed only 5–10% of the maximum activities observed at pH 10.0. Other ALDHs often show optical pH conditions at $\geqslant$8.0 [21–26]. Thus, the pH-dependent catalytic properties of PcALDHs were similar to those of other ALDHs.

Substrate specificity and kinetic parameters were further investigated using various aromatic aldehydes. Both rPcALDHs were capable of converting several aryl-aldehydes including vanillin, demonstrating broad substrate spectra (Table 2). Several studies on other organisms' ALDHs reported diagnostic substrate specificity against aryl-aldehyde compounds [27–29]. Compared with these ALDHs, the two PcALDHs showed comparatively similar activities against used substrates. The catalytic activities of PcALDH1 were significantly greater than those of PcALDH2. Taken together with the results from the proteomic differential display analysis, PcALDH1 would presumably play more crucial roles than PcALDH2 in fungal metabolism of various aryl-aldehydes. Interestingly, PcALDH1 showed 2-fold greater catalytic activity against benzoic acid than against other substrates, while PcALDH2 showed no substrate preferences among the aromatic aldehydes used in this study. This result suggests that PcALDH1 and PcALDH2 exhibit different substrate specificities.

Conclusions

We isolated two ALDHs from the white-rot basidiomycete *P. chrysosporium,* and showed that both converted various aryl-aldehydes to their corresponding aromatic acid compounds. Because a wide variety of aryl-aldehydes are generated as intermediates during the lignin degradation process, PcALDH1 and PcALDH2 need to be able to degrade a broad range of substrates

Table 2
Activity of the recombinant PcALDH1 and PcALDH2 towards aromatic aldehyde compounds.

Substrate	rPcALDH1[a]	rPcALDH2	Estimated reaction product
Benzaldehyde	0.268	0.071	Benzoic acid
3,4-Dihydroxybenzaldehyde	0.130	0.069	3,4-Dihydroxybenzoic acid
3,4-Dimethoxy-5-hydroxybenzaldehyde	0.123	0.061	3,4-Dimethoxy-5-hydroxybenzoic acid
3,4-Dimethoxybenzaldehyde	0.175	0.065	3,4-Dimethoxybenzoic acid
p-Hydroxybenzaldehye	0.124	0.064	*p*-Hydroxybenzoic acid
3,4,5-Trimethoxybenzaldehyde	0.144	0.064	3,4,5-Trimethoxybenzoic acid
4-Hydroxy-3-methoxybenzaldehyde	0.125	0.071	4-Hydroxy-3-methoxybenzoic acid
4-Hydroxy-3,5-dimethoxybenzaldehyde	0.099	0.055	4-Hydroxy-3,5-dimethoxybenzoic acid

Reactions were monitored by UV–vis spectrometer using 400 nm ($\Delta A400\ min^{-1}\ mL^{-1}$).
[a] Experiments were carried out in triplicate using 100 μL of purified enzyme (1.0 mg/mL) (SD $\leqslant$ 9%).

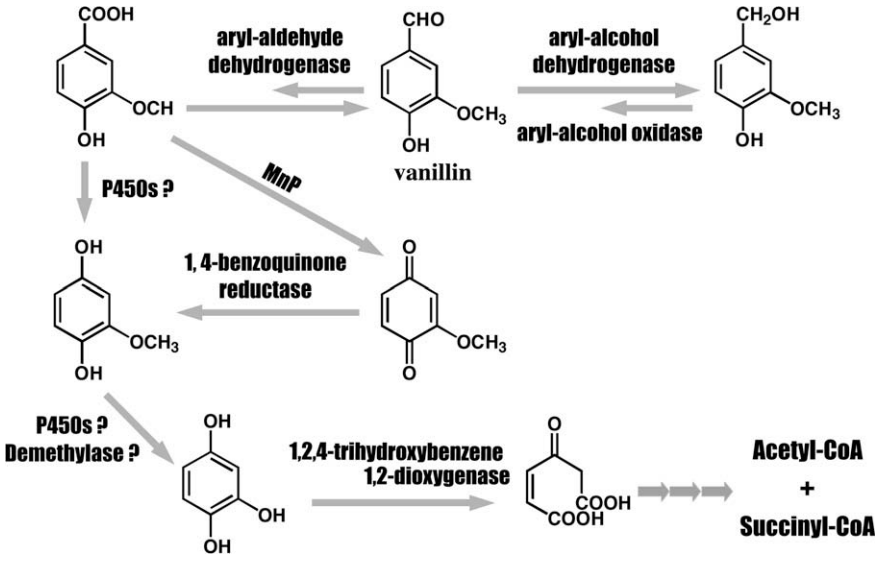

Fig. 4. Proposed metabolic pathway of vanillin by *P. chrysosporium*.

to provide sufficient metabolic diversity to degrade lignin efficiently. Compared with degradation of acid compounds, *P. chrysosporium* metabolizes aryl-aldehydes such as vanillin more rapidly to their corresponding alcohols [11]. However, once formed, the aromatic alcohol can be reoxidized to its corresponding aldehyde (Fig. 4). Thus, a reversible pathway from the aromatic acid to the aldehyde and then to the alcohol would be important for adaptation of metabolic flux and/or to detoxify these compounds [30,31]. Our results showed that PcALDH1 and PcALDH2 catalyzed oxidation of an aryl-aldehyde to its corresponding acid, but could not catalyze the reduction of an aromatic acid to its corresponding aldehyde. Therefore, up-regulation of PcALDH1 and PcALDH2 is a cellular response that activates an oxidative pathway for metabolism of aryl-aldehydes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.131.

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