Role of the Phenylalanine-Hydroxylating System in Aromatic Substance Degradation and Lipid Metabolism in the Oleaginous Fungus *Mortierella alpina*

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Mortierella alpina **is a filamentous fungus commonly found in soil that is able to produce lipids in the form of triacylglycerols that account for up to 50% of its dry weight. Analysis of the** *M. alpina* **genome suggests that there is a phenylalanine-hydroxylating system for the catabolism of phenylalanine, which has never been found in fungi before. We characterized the phenylalanine-hydroxylating system in** *M. alpina* **to explore its role in phenylalanine metabolism and its relationship to lipid biosynthesis. Significant changes were found in the profile of fatty acids in** *M. alpina* **grown on medium containing an inhibitor of the phenylalanine-hydroxylating system compared to** *M. alpina* **grown on medium without inhibitor. Genes encoding enzymes involved in the phenylalanine-hydroxylating system (phenylalanine hydroxylase [PAH], pterin-4α-carbinolamine dehydratase, and dihydropteridine reductase) were expressed heterologously in** *Escherichia coli,* **and the resulting proteins were purified to homogeneity. Their enzymatic activity was investigated by high-performance liquid chromatography (HPLC) or visible (Vis)-UV spectroscopy. Two functional PAH enzymes were observed, encoded by distinct gene copies. A novel role for tetrahydrobiopterin in fungi as a cofactor for PAH, which is similar to its function in higher life forms, is suggested. This study establishes a novel scheme for the fungal degradation of an aromatic substance (phenylalanine) and suggests that the phenylalanine-hydroxylating system is functionally significant in lipid metabolism.**

The phenylalanine-hydroxylating system catalyzes the irreversible hydroxylation of phenylalanine to tyrosine, which is the rate-limiting step in animal phenylalanine catabolism and protein and neurotransmitter biosynthesis and results in the formation of one molecule of fumarate and one of acetyl-coenzyme A (CoA) from each molecule of phenylalanine (1, 2). The animal phenylalanine-hydroxylating system consists of several essential components: phenylalanine hydroxylase (PAH) (EC 1.14.16.1), pterin-4α-carbinolamine dehydratase (PCD) (EC 4.2.1.96), dihydropteridine reductase (DHPR) (EC 1.5.1.34), and the obligatory cofactors tetrahydrobiopterin (BH$_4$) and molecular oxygen (3, 4) (Fig. 1). PAH is one of the BH$_4$-dependent aromatic amino acid hydroxylases, which also include tryptophan hydroxylase (TrpOHase) (EC 1.14.16.4) and tyrosine hydroxylase (TyrOHase) (EC 1.14.16.2) (5). BH$_4$ is regenerated following PAH-mediated phenylalanine hydroxylation by two additional enzymes, PCD and DHPR (Fig. 1) (6). The PAH, PCD, and DHPR genes responsible for the phenylalanine-hydroxylating system in animals have already been characterized (7, 8).

Phenylalanine is an important aromatic compound, one of the structurally diverse and second most abundant class of organic substrates (9). The greatest challenge for organisms using aromatic compounds as growth substrates is the stabilizing resonance energy of the aromatic ring system (10). This aromatic structure makes the substrate unreactive to oxidation or reduction and thus requires elaborate degradation strategies (9). The degradation of aromatic substances is dominated by aerobic and anaerobic bacteria and aerobic fungi, and the strategies used by these organisms are quite different from those of animals (10). Unlike higher or-

ganisms, most bacteria, fungi, and plants do not convert phenylalanine into tyrosine. The presence of PAH has been reported in only a few bacteria based on the identification of related genes (11–14). In the fungi *Aspergillus nidulans* and *Aspergillus fumigatus*, tyrosine was thought to be synthesized from phenylalanine by PAH (15, 16); however, no gene encoding PAH was found in their genomes (see Table S1 in the supplemental material). To date, no PAH, PCD, or DHPR involved in the phenylalanine-hydroxylating system has been identified in any fungi. Phenylalanine is converted into phenylacetate in *Penicillium chrysogenum*, *A. nidulans*, and *Aspergillus niger* (Fig. 2A) or into cinnamic acid in *Bjerkandera adusta* and *Schizophyllum commune* (Fig. 2C) (17–20). The cinnamate thus formed is converted to protocatechuate through benzoate, whereas phenylpyruvate is converted to homogentisate, which is catabolized by cleavage of the aromatic ring to yield fumarate and acetyl-CoA. Recently, we sequenced the whole genome of *Mortierella alpina* (ATCC 32222) (21), and our analysis suggested that there are two putative copies of the BH$_4$-dependent PAH gene for the catabolism of phenylalanine in *M. alpina*. Genes
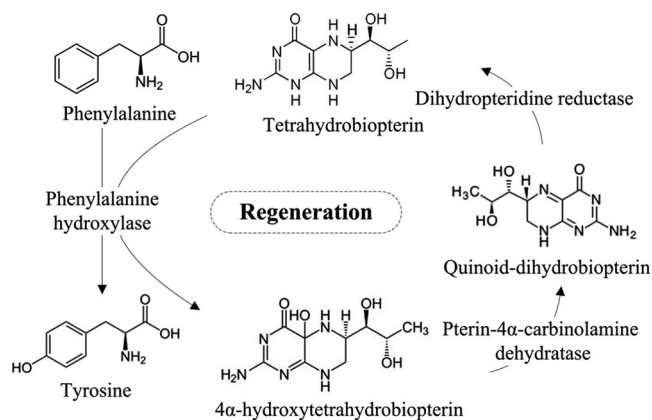
FIG 1 The animal phenylalanine-hydroxylating system.

encoding PCD and DHPR, which are required for the regeneration of BH₄, an essential component of the phenylalanine-hydroxylating system (6), were also found in the *M. alpina* genome. The BH₄ *de novo* synthesis pathway in *M. alpina* was first purified and characterized in our laboratory (22), but the function of BH₄ in fungi is still not well understood. The presence of the phenylalanine-hydroxylating system in *M. alpina* suggests that fungi can make use of the system in their phenylalanine degradation strategy.

M. alpina is a well-known polyunsaturated fatty acid (PUFA)-producing oleaginous fungus commonly found in soil (23). Some of the genes necessary for lipid synthesis in *M. alpina* have been cloned and partially characterized (24–28), and several biochemical reactions have been studied in detail (29, 30). However, the molecular mechanism of efficient lipid biosynthesis is still not well understood in oleaginous fungi in general and in *M. alpina* in particular. PAH and its cofactor BH₄ have been suggested to be essential for lipid metabolism in higher organisms (31–36), and some amino acid metabolism pathways have been postulated to be involved in fatty acid biosynthesis in oleaginous fungi (37, 38). However, the functional significance of the phenylalanine-hydroxylating system in the biosynthesis of lipids and closely related compounds has yet to be fully elucidated. In higher organisms, the phenylalanine-hydroxylating system is inhibited by *p*-chlorophenylalanine or esculin. Both are complete and irreversible inhibitors of PAHs *in vivo* (39, 40). *M. alpina* is noteworthy for its production of PUFA *de novo* and, due to its high lipid content (23), provides an interesting model for studying the relationship between the phenylalanine-hydroxylating system and lipid metabolism.

In this study, we investigated the role of the phenylalanine-hydroxylating system in lipid metabolism and probed its possible function in the degradation of aromatic substances. We established a novel role for BH₄ in fungi similar to that known to exist in higher life forms. We characterized the genes encoding PAH, PCD, and DHPR and their functions in the phenylalanine-hydroxylating system *in vitro*. We identified two functional PAH genes (the PAH-1 and PAH-2 genes) and measured kinetic parameters and the effects of temperature, pH, and aromatic amino acids on PAH activity. Multiple-sequence alignment and phylogenetic analysis of the PAH proteins with other, homologous proteins were also performed.

MATERIALS AND METHODS

Gene search. Predicted proteins in the *M. alpina* (ATCC 32222) genome (GenBank accession number ADAG00000000) were annotated by BLAST (41) searches against the following protein databases with the E value 1E−5: NR (http://www.ncbi.nlm.nih.gov), KOGs and COGs (42), KEGG
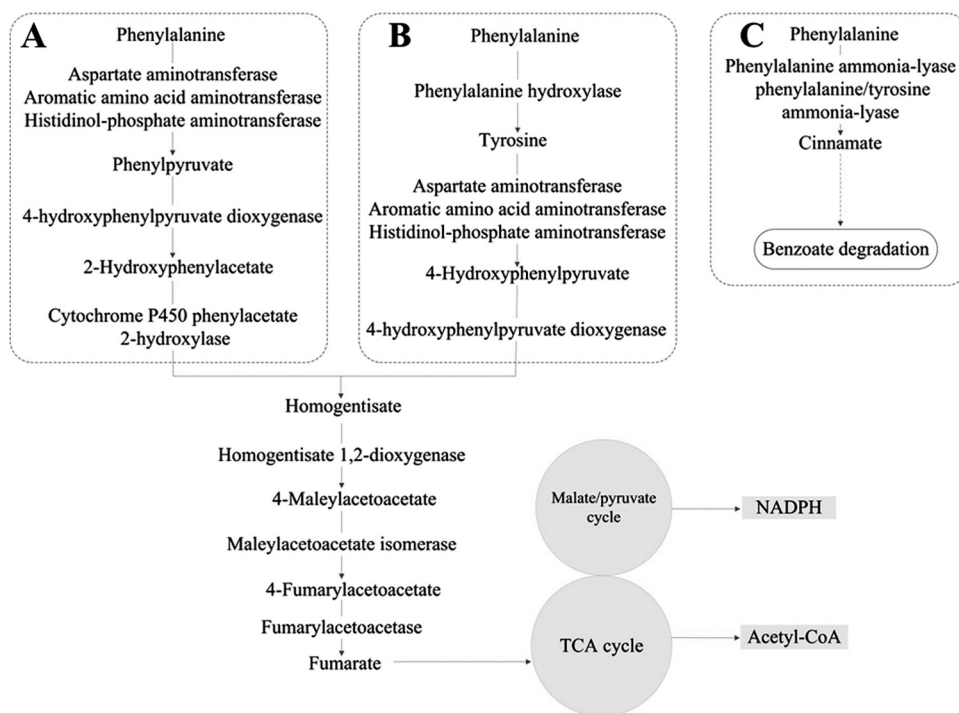

FIG 2 Fungal degradation of an aromatic substance (phenylalanine) in *P. chrysogenum*, *A. nidulans*, and *A. niger* (A), *M. alpina* (B), and *B. adusta* and *S. commune* (C). TCA, tricarboxylic acid.

(43), Swiss-Prot and UniRef100 (44), and BRENDA (45) and by Inter-ProScan (46) using the default parameter settings. Pathway mapping was conducted by associating the EC assignment and KO assignment with the KEGG metabolic pathways based on the BLAST search results. The predicted PAH-1, PAH-2, PCD, and DHPR proteins in the *M. alpina* genome were searched against predicted proteins of sequenced fungal genomes by BLAST with the E value 1E−5.

Strains and growth conditions. *M. alpina* (ATCC 32222) was cultured on potato dextrose agar at 25°C for 5 to 7 days. The fungal cultures were initially cultivated in 200 ml of Kendrick medium (47) and incubated at 25°C for 6 days. The mycelia were then collected by filtration through sterile cheesecloth and frozen immediately in liquid nitrogen for RNA extraction.

Aromatic compound degradation test. The indicated sole carbon sources (glucose at 3%, phenylalanine at 25 mM, phenylacetate at 10 mM, and tyrosine at 25 mM) (15) were added to minimal medium to test the aromatic compound degradation in *M. alpina*. The minimal medium contained 0.3% diammonium tartrate, 0.7% KH_2PO_4, 0.2% Na_2HPO_4, 0.15% $MgSO_4 \cdot 7H_2O$, 0.67% yeast nitrogen base, and 2% agarose, pH 6.0. The plates were photographed after 3 days of incubation at 25°C. The mycelia of *M. alpina* were stained with 0.01% triphenyltetrazolium chloride (TTC) to facilitate growth measurements (48). This experiment was replicated three times.

Effect of a PAH inhibitor on lipid synthesis. For the whole-cell inhibition studies, cultures were grown in 50 ml of Kendrick medium (50 g/liter glucose, 2 g/liter diammonium tartrate, 7.0 g/liter KH_2PO_4, 2.0 g/liter Na_2HPO_4, 1.5 g/liter $MgSO_4 \cdot 7H_2O$, 1.5 g/liter Bacto yeast extract, 0.1 g/liter $CaCl_2 2H_2O$, 8 mg/liter $FeCl_3 \cdot 6H_2O$, 1 mg/liter $ZnSO_4 \cdot 7H_2O$, 0.1 mg/liter $CuSO_4 \cdot 5H_2O$, 0.1 mg/liter $Co(NO_3)_2 \cdot 6H_2O$, and 0.1 mg/liter $MnSO_4 \cdot 5H_2O$, pH 6.0) with the addition of 5 mM *p*-chlorophenylalanine. The medium and incubation protocols were as previously described (21). The lipid composition of *M. alpina* was also investigated when *p*-chlorophenylalanine (5 mM) and tyrosine (5 mM) were both added to the medium. *M. alpina* cultures were incubated at 25°C for 6 days. *Aspergillus oryzae* is another oleaginous fungus (49, 50), and genome analysis suggests there is no phenylalanine-hydroxylating system in *A. oryzae* (see Table S2 in the supplemental material). The addition of PAH inhibitor in *A. oryzae* was included to verify whether there were any changes in lipids in the absence of a phenylalanine-hydroxylating system. *A. oryzae* was incubated at 25°C for 6 days. The mycelia were then collected by filtration, and approximately 20 mg was used for each lipid extraction. Accurately weighed portions of pulverized mycelium were extracted using the method of Bligh and Dyer (51) under acidified conditions with pentadecanoic acid and heneicosanoic acid added as internal standards (21). This experiment was replicated 3 times.

Cloning and plasmid construction. Total RNA extraction was performed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was subjected to RNase-free DNase digestion and then purified using the RNeasy Minikit (Qiagen). The quantity and quality of the total RNA were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). The total RNA was reverse transcribed with the PrimeScript RT reagent kit (TaKaRa Bio, Inc.) following the manufacturer's instructions before PCR amplification of PAH-1, PAH-2, PCD, and DHPR genes using the primer pairs shown in Table 1. The PCR conditions used were as follows: denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min (for 25 cycles in total). The final volume in each well was 50 μl. The amplified product was cloned into pET28a$^+$ to construct pwl47864 (containing PAH-1), pwl47866 (containing PAH-2), pwl39696 (containing PCD), or pwl39698 (containing DHPR). The presence of inserts in the plasmids was confirmed by sequencing using an ABI 3730 sequencer.

Protein expression and purification. *Escherichia coli* BL21 carrying pwl47864, pwl47866, pwl39696, or pwl39698 was grown overnight at 37°C with shaking in LB medium containing 50 μg/ml of kanamycin. The overnight culture (10 ml) was inoculated into 500 ml of fresh medium and

TABLE 1 Primers used in the study

Primer	Primer sequence used for cloning[a]
PAH-1 (forward)	CGGAATTCATGTCTGCTGCTGCCAACCACCT
PAH-1 (reverse)	CCCAAGCTTTTACGCCAGCTTCTGCAGGGC
PAH-2 (forward)	CGGAATTCATGTCCTCCACCCCAGGCC
PAH-2 (reverse)	CCCAAGCTTTTACATCTTCTGCAGGGCATCGAC
PCD (forward)	CGGAATTCATGACTCTCAAGAAGCTCGATG
PCD (reverse)	CCGCTCGAGTTATTGCTTGAAGATCTCATCG
DHPR (forward)	CGGAATTCATGGTTTCTAGCATCGTCGTCTAT
DHPR (reverse)	CCGCTCGAGTTACTTCTCGGTGTACGTGGTGT

[a] Restriction enzyme cleavage sites are underlined.

grown until the optical density at 600 nm (OD_{600}) reached 0.6. PAH-1 expression was induced by the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 30°C for 20 h, PAH-2 expression was induced by 0.01 mM IPTG at 30°C for 20 h, and PCD or DHPR expression was induced by 0.5 mM IPTG at 37°C for 16 h. After the IPTG induction, cells were harvested by centrifugation, washed with binding buffer (50 mM Tris-HCl, 300 mM NaCl, and 10 mM imidazole; pH 8.0), resuspended in the same buffer (supplemented with 1 mM phenylmethanesulfonyl fluoride and 1 mg/ml lysozyme), and then sonicated. Cell debris was removed by centrifugation, and the supernatants (containing the soluble proteins) were collected. The His-tagged fusion proteins were purified by nickel ion affinity chromatography using a Chelating Sepharose Fast Flow column (GE Healthcare) according to the manufacturer's instructions. Unbound proteins were washed through with 100 ml of wash buffer (50 mM Tris-HCl, 300 mM NaCl, and 25 mM imidazole, pH 8.0). Fusion proteins were eluted with 3 ml of elution buffer (50 mM Tris-HCl, 300 mM NaCl, and 250 mM imidazole, pH 8.0) and dialyzed overnight at 4°C against 50 mM Tris-HCl buffer containing 20% glycerol (pH 7.4). The protein concentrations were determined by the Bradford method. The purified proteins were stored at −80°C.

Enzyme activity assays. The PAH activity was assayed using the method of Bailey and Ayling (52) with minor modifications. The reaction mixture for PAH contained 100 mM Tris-HCl (pH 7.0), 1 mM phenylalanine, 1 mg/ml catalase, and 0.14 μg/ml purified PAH-1 protein or 0.22 μg/ml purified PAH-2 protein and was maintained at 25°C for 5 min. Then, 100 μM ferrous ammonium sulfate was added and allowed to incubate for 1 min. The reaction was started by the addition of 100 μM BH_4 (Sigma) or 6-methyltetrahydropterin (Sigma) and 5 mM dithiothreitol (DTT) in a total volume of 50 μl. After a period of 15 min, the reaction was stopped by adding 50 μl 2 M trichloroacetic acid. Samples were prepared for high-performance liquid chromatography (HPLC) after centrifugation. The HPLC was performed on a Shimadzu model LC-20AT equipped with a Rheodyne (IDEX Health and Science) loop of 20 μl, an Inertsil ODS-3 column (5 μm; 150 by 4.6 mm; GL Science), and a Shimadzu model RF-20A fluorescence detector. The mobile phase consisted of 0.1 M NH_4OH adjusted to pH 4.6 with acetic acid at a flow rate of 1.5 ml/min. To observe the separation of phenylalanine and tyrosine, the excitation and emission wavelengths of the fluorescence detector were set at 260 and 282 nm, respectively (53). To determine the amount of tyrosine, the excitation and emission wavelengths of the fluorescence detector were set at 274 and 304 nm, respectively (52). Quantitation was based on external calibration using a standard curve prepared with different amounts of tyrosine.

The reaction mixture for DHPR contained 100 mM Tris-HCl (pH 7.0), 10 μg/ml peroxidase, 10 mM hydrogen peroxide, 10 μM BH_4, and 0.1 mM NADH. After an incubation period of 4.5 min at 25°C, the reaction was started by adding 3.99 μg/ml purified DHPR protein in a final volume of 1 ml. The initial rates were obtained from the initial rate of decrease of A_{340} (E_{340} for NADH, 6,200 M^{-1} cm^{-1}) (54, 55). The background reactions were eliminated when the reaction was catalyzed by peroxidase or DHPR alone.

The 50-μl PCD assay reaction mixture contained 100 mM Tris-HCl (pH 7.0), 1 mg/ml catalase, 1 mM L-phenylalanine, and 0.14 μg/ml puri-

fied PAH-1 protein. The mixture was incubated for 2 min at 25°C, and 20 μM BH$_4$ and 5 mM DTT were added. After a 5-hour PAH reaction, 0.1 mM NADH, 6.24 μg/ml purified PCD protein, and 3.99 μg/ml purified DHPR protein were added. The amount of tyrosine formed after 24 h was determined fluorometrically and corrected for the tyrosine formed in controls without PCD (56, 57). The background reactions were eliminated when the reaction was catalyzed by PCD or DHPR alone. These experiments were replicated 3 times.

Determination of the temperature and pH optima and effects of aromatic amino acids on PAH activity. In order to determine the optimum temperature, PAH reactions were carried out at 5°C, 15°C, 25°C, 37°C, 50°C, and 60°C for 15 min at pH 7.0. For determinations of optimum pH, three different buffers were used over a range of pH 3 to 11, including 100 mM sodium acetate-acetic acid (pH 3 to 6), 100 mM Tris-HCl (pH 7 to 8), and 100 mM glycine-NaOH (pH 10 to 12). These reactions were carried out for 15 min at 25°C. A range of amino acid concentrations (0.5 μM to 5 mM) were used to test their effects on the PAH activity, including tryptophan, glycine, alanine, p-chlorophenylalanine, and esculin. These reactions were carried out for 15 min at 25°C, pH 7.0. The experiments were replicated 3 times.

Measurement of kinetic parameters. To measure the K_m and V_{max} values for both PAHs using phenylalanine, reactions were carried out using various concentrations of phenylalanine (0.5 to 2 mM) in conjunction with a fixed concentration of BH$_4$ (1 mM). To measure the K_m and V_{max} values for both PAHs acting on BH$_4$, reactions were carried out using various concentrations of BH$_4$ (70 to 400 μM) but with a fixed concentration of phenylalanine (1 mM). The PAH reactions were performed in Tris-HCl, pH 7.5, at 25°C for 15 min in a final volume of 50 μl. The conversion of phenylalanine to tyrosine was monitored by HPLC. The kinetic parameters were obtained by fitting the initial rates of reaction measured at various concentrations of one substrate at a fixed concentration of the other to the Michaelis-Menten equation using nonlinear regression. This experiment was replicated 3 times.

RESULTS

Gene search. Putative PAH-1, PAH-2, PCD, and DHPR genes were found in the *M. alpina* genomes (GenBank accession numbers JN982953, JN982954, JQ281474, and JQ281475), suggesting the presence of a phenylalanine-hydroxylating system in the fungus. The search results for all of the sequenced fungal genomes are shown in Table S1 in the supplemental material.

Aromatic compound degradation test. The growth phenotypes of *M. alpina* are shown in Fig. S1 in the supplemental material. *M. alpina* showed fine hyphal growth on medium containing phenylalanine (see Fig. S1B in the supplemental material) or tyrosine (see Fig. S1E) as the sole carbon source. The growth of *M. alpina* was repressed by the addition of p-chlorophenylalanine to medium containing phenylalanine as the sole source of carbon (see Fig. S1D). *M. alpina* was unable to grow on medium containing phenylacetate as the sole carbon source (see Fig. S1C), even if it was incubated for over 2 weeks or at a higher phenylacetate concentration (1 M), which is in contrast to other fungi, such as *P. chrysogenum*, *A. nidulans*, and *A. niger* (17–20). Thus, these results show that *M. alpina* can degrade phenylalanine and tyrosine, but not phenylacetate.

Effects of p-chlorophenylalanine on PUFA synthesis. To explore the relationship between the phenylalanine-hydroxylating system and lipid synthesis, we investigated the effects of 5 mM p-chlorophenylalanine, an inhibitor of the phenylalanine-hydroxylating system in higher organisms, on the fatty acid status (see Table S2 in the supplemental material). The *M. alpina* cultures grown in medium plus inhibitor showed reduced total saturated fatty acids (SAFA), ω6 PUFA, ω3 PUFA, and total fatty acid
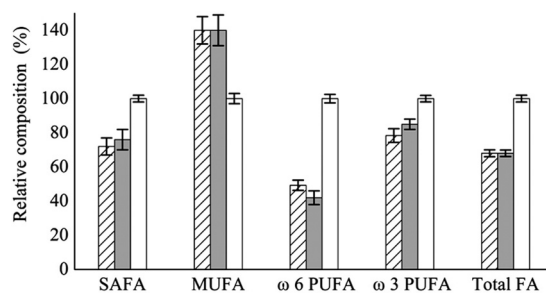

FIG 3 Effects of an inhibitor (p-chlorophenylalanine) of the phenylalanine-hydroxylating system on fatty acid synthesis in *M. alpina*. The hatched bars, shaded bars, and open bars represent *M. alpina* grown in inhibitor medium, inhibitor and tyrosine medium, and inhibitor-free medium, respectively. The data were normalized to the concentration of fatty acids (mg/g [cell dry weight]) from *M. alpina* grown on inhibitor-free medium, defined as 100%. The data shown are the averages ($\pm$ standard deviations) of three independent experiments.

(FA) accumulation levels by about 30%, 50%, 20%, and 30%, respectively, while the level of monounsaturated fatty acid (MUFA) was increased by about 30% (Fig. 3). The addition of tyrosine did not change the inhibitory effects of p-chlorophenylalanine on lipids when *M. alpina* was grown in medium plus inhibitor and tyrosine (Fig. 3). The lipid status of *A. oryzae* grown on PAH inhibitor medium did not change compared to medium without inhibitor (see Table S2 in the supplemental material), suggesting that the PAH inhibitor used in our experiment is selective.

Expression and purification of PAHs, PCD, and DHPR. PAH-1, PAH-2, PCD, and DHPR were expressed as His-tagged fusion proteins in *E. coli* BL21 by IPTG induction and purified to near homogeneity by nickel ion affinity chromatography (see Fig. S2 in the supplemental material). The molecular masses estimated from the SDS-PAGE analysis were 54 kDa for PAH-1 and PAH-2, 14 kDa for PCD, and 27 kDa for DHPR, which correspond well to the calculated masses (excluding the His tag, these were 50 kDa, 50 kDa, 11 kDa, and 23 kDa, respectively). The yields of PAH-1, PAH-2, PCD, and DHPR were 1.40 μg/ml, 2.24 μg/ml, 62.44 μg/ml, and 39.88 μg/ml, respectively.

Activities of PAHs, PCD, and DHPR. Phenylalanine was incubated with the purified PAH proteins, and the reaction products were identified by HPLC, as shown in Fig. S3 in the supplemental material. Phenylalanine was observed at 6.5 min, corresponding to the position of the phenylalanine standard (see Fig. S3B in the supplemental material) in the sample incubated in the absence of the PAHs (see Fig. S3C). Phenylalanine was converted to a peak that eluted at 2.6 min, corresponding to the position of the tyrosine standard (see Fig. S3A) in the reaction catalyzed by PAH-1 (see Fig. S3D) or PAH-2 (see Fig. S3E). These results confirm that the recombinant proteins show PAH activity, which is dependent on the presence of all of the substrates (including BH$_4$ and 6-methyltetrahydropterin). To examine whether the purified DHPR protein showed DHPR activity, we determined the initial velocity of its NADH consumption. When the reaction was catalyzed by peroxidase or DHPR alone, it had no effect on the rate of NADH oxidation. The initial rate of NADH oxidation was dramatically increased by the addition of the recombinant protein, indicating that the purified protein possesses DHPR activity (see Fig. S4 in the supplemental material). The specific activity of the
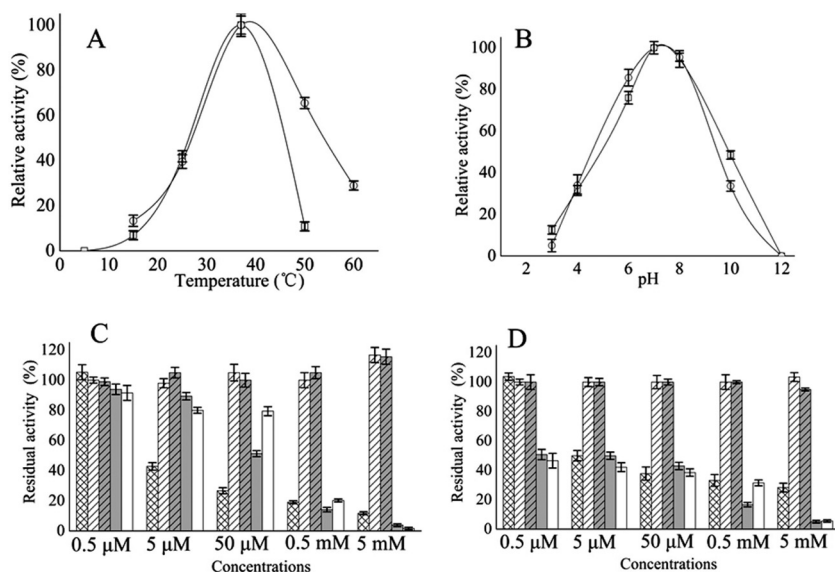
FIG 4 Effects of temperature (A), pH (B), and aromatic amino acids (C and D) on PAH-1 (A and B, open circles, and C) and PAH-2 (A and B, open squares, and D) activity. The crosshatched bars, open hatched bars, shaded hatched bars, shaded bars, and open bars show the effects of tryptophan, glycine, alanine, *p*-chlorophenylalanine, and esculin, respectively, on PAH activity. Each point represents the average (± standard deviation) of three independent measurements.

recombinant DHPR protein was 29.92 μmol/min/mg. One unit of DHPR activity was defined as being the amount of enzyme that caused the oxidation of 1 μmol NADH per minute under the above conditions. PCD activity was assayed by the ability to stimulate PAH activity in the presence of BH_4. When the reaction was catalyzed by PCD or DHPR alone, it had no effect on the rate of tyrosine production. The amount of tyrosine formed with the addition of PCD was 0.17 mmol, and the tyrosine formed in controls without PCD was 0.13 mmol. Thus, the specific activity of the recombinant PCD protein was 0.35 μmol/min/mg. One unit of PCD activity is defined as 1 μmol of tyrosine formed in 30 min under the above conditions. The data shown are the averages of three independent experiments.

Effects of temperature, pH, and aromatic amino acids on PAH activity. PAH-1 and PAH-2 were active at each of the temperatures tested (in the range of 15°C to 65°C), with the greatest activity detected at 37°C and 40°C (Fig. 4A). One hundred percent activity in the PAH-1 and the PAH-2 reactions occurred at 37°C under the aforementioned conditions. The optimal temperatures for PAH-1 and PAH-2 are in accordance with data previously obtained for humans and *Dictyostelium discoideum* (58, 59) but are different from data obtained for *Chromobacterium violaceum*, *Colwellia psychrerythraea*, and *Chloroflexus aurantiacus* (13, 60, 61). PAH-1 activity decreased quickly at higher temperatures and was limited at 50°C (Fig. 4A). For PAH-2, high activity (65%) was observed at temperatures up to 50°C, and activity was still found at 60°C (Fig. 4A). Therefore, PAH-2 was more heat stable than PAH-1.

PAH-1 and PAH-2 both showed optimal activity at pH 7.5 in 100 mM Tris-HCl buffer (Fig. 4B), which is similar to the optimum pH for recombinant human and *C. violaceum* PAHs (59, 60). Maximal activity for both PAH-1 and PAH-2 occurred at pH 7.0 under the aforementioned conditions. The PAHs were active over a broad range of pH values (4 to 10), which contrasts with the significantly decreased activity of human PAH below pH 5.5. PAH-1 and PAH-2 exhibited a very similar pH sensitivity profile.

Some aromatic amino acids react with the substrate binding site of PAH and can be hydroxylated under appropriate conditions *in vitro* or can competitively inhibit the hydroxylation of phenylalanine (59). PAH-1 activity was significantly inhibited by 5 mM concentrations of a variety of amino acids, including tryptophan (90% inhibition) and *p*-chlorophenylalanine (96% inhibition) (Fig. 4C). PAH-1 activity was also significantly inhibited by 5 mM esculin (98% inhibition) (Fig. 4C). The inhibition profile of PAH-2 activity was similar: tryptophan (70% inhibition), *p*-chlorophenylalanine (95% inhibition), and esculin (95% inhibition) (Fig. 4D). However, low concentrations of *p*-chlorophenylalanine and esculin were more effective in reducing PAH-2 than PAH-1 activity. Glycine and alanine had little effect on PAH-1 and PAH-2 activity. One hundred percent activity was defined as a PAH reaction without competing amino acids under the previous PAH activity assay conditions. The inhibitory effects of the aromatic amino acids and esculin were also observed for PAH in humans, and this inhibition is one of the hallmarks of PAH activity (59).

Comparative analysis of *M. alpina* PAH proteins and other homologous proteins. The kinetic parameters of PAH-1 and PAH-2 (for phenylalanine and BH_4) were measured. The kinetics of the reactions catalyzed by these enzymes present a good fit with the Michaelis-Menten model, and nonlinear regression of the experimental data to the Michaelis-Menten equation yielded the kinetic parameters shown in Table 2. PAH-1 and PAH-2 share 12.7 to 55.3% identity with other characterized PAH proteins and

TABLE 2 Kinetic parameters[a]

Enzyme	Substrate	K_m (mM)	V_{max} (μM/min)
PAH-1	Phenylalanine	0.1750 ± 0.003962	3.827 ± 0.1342
PAH-1	BH_4	0.1253 ± 0.002518	2.176 ± 0.03927
PAH-2	Phenylalanine	0.06885 ± 0.002018	11.21 ± 0.2164
PAH-2	BH_4	0.04778 ± 0.001889	5.879 ± 0.06391

[a] The data shown are the averages of three independent experiments.

are most similar to those from *D. discoideum*. Multiple-sequence alignment of the *M. alpina* PAHs and other characterized PAH proteins revealed the presence of certain conserved motifs and residues (see Fig. S5 in the supplemental material), including two motifs, Gly-Ala-Leu (residues 46 to 48 in human PAH) and Ile-Glu-Ser-Arg-Pro (residues 65 to 69 in human PAH), that form a phenylalanine-binding pocket (62); a Gly-Leu-Leu-Ser-Ser motif (residues 247 to 251 in human PAH) involved in BH_4 cofactor binding (13, 63, 64); two motifs, Tyr-Thr-Pro-Glu-Pro (residues 277 to 281 in human PAH) and Gly-Ala-Gly-Leu-Leu-Ser (residues 344 to 350 in human PAH), involved in substrate binding (13, 64); 4 iron-binding residues (human His146, Arg158, His285, and His290) (65–67); 4 residues (human Arg270, Trp326, Phe331, and Glu353) involved in substrate binding (13, 64); 6 residues (human Phe254, Leu255, His264, Glu286, Ala322, and Tyr325) involved in BH_4 cofactor binding (13, 63, 64); and 6 residues (human Pro122, Leu128, Phe131, Ala132, Arg158, and His146) involved in the N-terminal regulatory domain. The residues of the pterin binding loop, the N-terminal loop, and the loop region between residues 272 and 282 differ from bacterial and human versions of PAH, creating a 10-fold-higher specific activity for BH_4 in *C. violaceum* than in humans (65, 68). These residues in human PAH are almost all conserved in *M. alpina* PAH, except for human Ile125, Ile135, and Gly272 and the N-terminal autoregulatory sequence (NARS) (human residues 19 to 33) (65), which is consistent with the similar activity observed in human PAH and *M. alpina* PAHs. Residues Asp116, Lys274, and Ser439, which are responsible for dimer and tetramer formation, substrate binding, and deamidation rates, respectively (65, 67), are not conserved in PAH-1 but do occur in PAH-2. The increased K_m of *M. alpina* PAH-1 compared with PAH-2 may be due to the loss of one or several of these residues, which may consequently impair substrate binding and decrease catalytic activity. The other enzymatic properties of PAH-1 and PAH-2 are generally similar, which is consistent with the data observed for eukaryotes (58, 59).

Phylogenetic analysis of PAH. A phylogenetic tree was generated with amino acid hydroxylase (AAH) sequences from fungi, animals, protists, plants, and bacteria (see Fig. S6 in the supplemental material). The tree is rooted with PAHs from plant species as an outgroup and further recognizes PAH as an ancestral AAH function and gives insight into the evolution of AAHs (58, 69). Based on the search results in the fungal genomes (see Table S1 in the supplemental material), *M. alpina*, *Allomyces macrogynus*, *Batrachochytrium dendrobatidis*, *Rhizopus oryzae*, *Spizellomyces punctatus*, *Mucor circinelloides*, and *Phycomyces blakesleeanus* are fungi that contain a putative PAH gene. The BH_4 de novo pathway is conserved in all of these fungi except *A. macrogynus*, due to the absence of the 6-pyruvoyltetrahydropterin synthase gene (EC 4.2.3.12) (see Table S1 in the supplemental material). PAH cannot work in *A. macrogynus* due to the lack of cofactor BH_4, which may be why *A. macrogynus* PAH and other fungal PAHs cluster as a distinct group in the phylogenetic tree. Our phylogenetic analysis of fungal PAH sequences and animal, protist, plant, and bacterial AAHs of known specificity places the fungal proteins closest to those of animals. The tree illustrates the clear distinction between fungi that contain the phenylalanine-hydroxylating system and those that do not and also shows the close relationship between *M. alpina* and animal PAH (see Fig. S6 in the supplemental material). Based on sequence comparisons and enzymatic properties, the

PAHs from *M. alpina* and animals are thought to have evolved from the same ancestor.

DISCUSSION

The fungal BH_4 synthesis pathway has been characterized at the molecular level only in *M. alpina* (22), and BH_4 acts as a cofactor in nitric oxide synthesis only in the fungi *P. blakesleeanus* and *Neurospora crassa* (70, 71). Because most fungi do not synthesize BH_4 and lack BH_4-dependent monooxygenases, the function of BH_4 in fungi is unclear. The BH_4-dependent PAH is present in only about 7% of the fungal genomes sequenced so far (see Table S1 in the supplemental material), and other BH_4-dependent enzymes have not been discovered in *M. alpina*. This work provides direct biochemical evidence that BH_4 can function as a biological cofactor for PAH in fungi, based on the characterization of the BH_4 synthesis pathway and PAH homologues in *M. alpina*. This function has never been demonstrated in fungi before, and thus, we hypothesize that BH_4 plays a novel role in fungi similar to that known to exist in higher life forms.

In most fungi, phenylalanine is converted into phenylacetate. The conventional aerobic route for phenylacetate, which leads to homogentisate, occurs in fungi (Fig. 2) (17–20). However, the *M. alpina* genome sequence lacks an ortholog of phenylacetate 2-hydroxylase (EC 1.14.13.-) to catalyze the hydroxylation of phenylacetate to homogentisate (Fig. 2B), which is consistent with the inability of *M. alpina* to grow on medium containing phenylacetate as the sole carbon source (see Fig. S1C in the supplemental material). This is in contrast to the fact that most fungi can grow on phenylacetate medium (15). Because this step is needed for the complete oxidation of phenylalanine to carbon dioxide and water (14), conventional fungal phenylalanine catabolism is blocked in *M. alpina*. However, *M. alpina* can grow on phenylalanine as the sole carbon source (see Fig. S1B in the supplemental material), which means that it has an unconventional strategy for phenylalanine metabolism. In this report, we have shown that in this organism, phenylalanine is hydroxylated to tyrosine by the phenylalanine-hydroxylating system; tyrosine can then be converted into 4-hydroxyphenylpyruvate, followed by hydroxylation to homogentisate, the same intermediate as in other fungi (Fig. 2). The putative genes needed for tyrosine oxidation are all present in the *M. alpina* genome, which was proved by the fact that *M. alpina* could grow on tyrosine as the sole carbon source (see Fig. S1E in the supplemental material). Our characterization of the phenylalanine-hydroxylating system provides the molecular basis for a novel scheme for the degradation of an aromatic substance (phenylalanine) in fungi. This paradigm for phenylalanine catabolism differs significantly from the established knowledge for other fungi. Conserved genes of the phenylalanine-hydroxylating system have been found in 7% of the fungal species sequenced thus far (see Table S1 in the supplemental material). These species belong to the groups of mucoromycotina (*M. alpina*, *R. oryzae*, *M. circinelloides*, and *P. blakesleeanus*) and chytridiomycota (*B. dendrobatidis* and *S. punctatus*). Thus, this scheme for aromatic degradation is of major importance but is not widespread in fungi.

In higher organisms, PAH and its cofactor BH_4 are suggested to be essential for lipid metabolism. Mutations in the human PAH gene result in the metabolic disorder known as phenylketonuria (PKU) (67), and PKU patients present with lower concentrations of long-chain polyunsaturated fatty acids (LCPUFA), such as arachidonic acid (AA) and eicosapentaenoic acid, and higher con-

centrations of oleic acid 18:1 (31, 32). It has also been suggested that the PAH cofactor BH_4 is essential for the desaturation or omega oxidation of long-chain fatty acids and the incorporation of unsaturated fatty acids into phospholipids (33–36). Significant changes were observed in the concentration of total ω6 PUFA and AA in *M. alpina* grown on PAH inhibitor medium compared to *M. alpina* grown on medium without inhibitor (Fig. 3). Of all the fatty acids, AA—the main commercial product of *M. alpina* (23)—showed the most pronounced change. Interestingly, the trends in the lipid status of *M. alpina* grown on PAH inhibitor medium parallel those observed in PKU patients. These results suggest that the phenylalanine-hydroxylating system may control lipid accumulation, either on its own or via interactions with other as yet unrecognized regulatory factors in *M. alpina*. Acetyl-CoA and NADPH are two key factors in lipid metabolism. Acetyl-CoA is the essential building block and necessary precursor of fatty acids (30), and NADPH is the critical reducing agent and limiting factor in fatty acid synthesis (72). Based on the genome information, the phenylalanine catabolism pathway in *M. alpina* was constructed to map the utilization of phenylalanine (Fig. 2). The *M. alpina* phenylalanine-hydroxylating system is supposed to be blocked by PAH inhibitor, which may interfere with fatty acid synthesis by reducing the production of NADPH and acetyl-CoA through the tricarboxylic acid cycle. We hypothesize that the function of the phenylalanine-hydroxylating system in *M. alpina*, including PAH and BH_4, is to contribute NADPH and acetyl-CoA for lipid accumulation by the utilization of phenylalanine and tyrosine, and this may also explain in part how *M. alpina* achieves its high level of lipid synthesis. Moreover, as there are two copies of PAH in *M. alpina* and PAH is the rate-limiting factor in animal phenylalanine catabolism (1, 2), ectopic overexpression of PAH should enhance the ability of *M. alpina* to use phenylalanine for lipid synthesis. RNA interference (RNAi) of PAH genes should be utilized in future work to explore the relationship between phenylalanine-hydroxylating system and lipid synthesis.

It was suggested that NADPH produced by malic enzymes was the unique source of reducing power for fatty acid synthesis in fungi like *M. circinelloides* and *M. alpina* and that malic enzyme is consequently the rate-limiting step for fatty acid biosynthesis (73). Recently, leucine metabolism was found to be another bottleneck in lipid accumulation in the oleaginous fungus *M. circinelloides* (37). In that work, the overexpression of the LEU2 gene (a gene encoding β-isopropylmalate dehydrogenase, involved in leucine biosynthesis) significantly increased the lipid content, in accordance with previous observations in *Saccharomyces cerevisiae* (74). Comparative genome analysis of the nonoleaginous and oleaginous strains points to a relationship between lipid and amino acid metabolism, including leucine and lysine degradation and lysine biosynthesis, in the biosynthesis of the important intermediate metabolite acetyl-CoA, which might be related to the capability of the oleaginous microorganisms to accumulate high levels of lipids (38). Leucine and lysine metabolic pathways were all conserved in the *M. alpina* genome. Our study suggests that phenylalanine metabolism in *M. alpina* may also be important for lipid accumulation. It may support previous data postulating amino acid metabolism as one of the pathways involved in the generation of the acetyl-CoA required for fatty acid biosynthesis (38, 74). The relationship between lipid and amino acid metabolism is thus of major importance and is probably widespread in oleaginous fungi. Our identification of a phenylalanine-hydroxylating system in-

volved in amino acid metabolism and lipid accumulation extends the range of target candidates for genetic manipulation in *M. alpina* for obtaining strains with increased amounts of lipids.

In conclusion, the phenylalanine-hydroxylating system has been characterized at the molecular level in a fungus, *M. alpina*. Genes encoding PAH, PCD, and DHPR were functionally characterized *in vitro*. Two functional PAH genes (the PAH-1 and PAH-2 genes) were observed. A novel role for tetrahydrobiopterin in fungi as a cofactor for PAH similar to that known to exist in higher life forms is suggested. This work establishes a novel scheme for the fungal degradation of an aromatic substance (phenylalanine) and suggests that the phenylalanine-hydroxylating system is functionally significant in lipid metabolism.

ACKNOWLEDGMENTS

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