Atypical Biosynthetic Properties of a $\Delta^{12}/\nu+3$ Desaturase from the Model Basidiomycete *Phanerochaete chrysosporium*[†]

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The model white-rot basidiomycete *Phanerochaete chrysosporium* contains a single integral membrane Δ^{12} -desaturase FAD2 related to the endoplasmic reticular plant FAD2 enzymes. The fungal *fad2*-like gene was cloned and distinguished itself from plant homologs by the presence of four introns and a significantly larger coding region. The coding sequence exhibits ca. 35% sequence identity to plant homologs, with the highest sequence conservation found in the putative catalytic and major structural domains. In vivo activity of the heterologously expressed enzyme favors C_{18} substrates with $\nu+3$ regioselectivity, where the site of desaturation is three carbons carboxy-distal to the reference position of a preexisting double bond (ν). Linoleate accumulated to levels in excess of 12% of the total fatty acids upon heterologous expression of *P. chrysosporium* FAD2 in *Saccharomyces cerevisiae*. In contrast to the behavior of the plant FAD2 enzymes, this oleate desaturase does not 12-hydroxylate lipids and is the first example whose activity increases at higher temperatures (30°C versus 15°C). Thus, while maintaining the hallmark activity of the fatty acyl Δ^{12} -desaturase family, the basidiomycete *fad2* genes appear to have evolved substantially from an ancestral desaturase.

Desaturases, the enzymes responsible for unsaturated fatty acid biosynthesis, are found throughout the eukaryotic taxa. Critical cellular processes dependent on the modification of acyl lipids by desaturases include the regulation of membrane structure and fluidity, proper function of ion channels and other membrane proteins, and the biosynthesis of signaling molecules, such as jasmonic acid and arachidonic acid-derived second messengers (53, 71). Polyunsaturated fatty acids (PUFAs) with double bonds at carbon-12, such as linoleic acid (18:2 Δ^9 ,12c), are not synthesized by animals, who therefore depend upon the activities of the stepwise action of the Δ^9 - and Δ^{12} -desaturases from plants and lower eukaryotes to generate these essential lipids.

Supplementation of our diet with PUFAs derived from transgenic organisms has been targeted in recent years. Expression of fungal (37) and plant (56) desaturase genes in mammalian cells has been explored as a means to enhance the nutritional quality of meat products. Oleate and PUFA desaturases and elongases are gene targets sought after for transgenic production of the C_{20} and C_{22} polyunsaturated food supplements docosahexenoic and eicosapentenoic acids in algae, plants, and yeast (35, 51). The practical success of lipid metabolic engineering studies is dependent upon the expression of enzymes with high chemo- and regioselectivity within the transgenic organism, coupled with the manipulation of lipid biochemical flux to result in high, economically viable levels of unsaturated storage oil accumulation.

Two evolutionarily distinct desaturase types exist: the soluble plastidial and the membrane-bound endoplasmic reticulum

(ER)-localized enzymes, both of which use NAD(P)H and O_2 to sequentially abstract two hydrogens from vicinal sp^3 -hybridized carbons leading to a *cis*-alkene (15, 61). Current models for all fatty acyl desaturases postulate the activation of molecular oxygen at a nonheme diferrous active site that culminates with two C-H bond scissions and the formation of water (20, 29, 39, 46). In the case of the microsomal desaturases, conserved iron ligands appear to be located in three distinctive histidine box motifs (61). Microsomal Δ^{12} -desaturases (FAD2s) are best known from plants, where they exhibit substantial (60 to 90%) sequence identity and have focused regioselectivity yet have evolved into the diverged desaturases that catalyze distinct oxidative processes, resulting in natural products with hydroxy, conjugated polyalkenyl, epoxy, and acetylenic functionalities (65). Studies of the FAD2 superfamily have been propelled by commercial interest in the modification of standard oilseed crops with “diverged” *fad2* genes that show atypical regio- and/or chemoselectivity (32, 38, 50). Despite the sheer number of plant Δ^{12} -desaturases, the refractory nature of these membrane-bound enzymes to purification has left structure/function relationships ill defined (33). Consequently, only the rough classification of the FAD2 enzymes into distinct functional or evolutionary classes has occurred, largely using genetic and in vivo functional characterization (12, 38, 42).

While the Δ^{12} - or FAD2 desaturases, which form a 12,13-double bond, are best known from plants, fungal *fad2* homologs have been found in zygomycetes and ascomycetes (8, 52, 59). As suggested by molecular clock data, *Basidiomycota* and *Ascomycotina* diverged approximately 550 million years ago (3), indicating that metabolic basidiomycete genes may differ significantly from those of other fungal subtypes. With their high linoleic acid content, typically 60 to 80% of the lipid in basidiomycete fruiting bodies (63), and their ability to grow under varied temperature regimes, macrofungi provide an untapped genetic resource for desaturases that may be well suited

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for biotechnological applications. Indeed, two homobasidiomycete Δ^{12} -desaturases have been recently reported (57, 77).

In fungi, variations in membrane lipid composition caused by temperature cycling may be integral to the morphological changes of fruit body formation (58). Linoleate-derived hydroxy fatty acids and lactones have been shown to provide molecular signals, called Psi factors, involved in ascomycete sporulation (8, 9). Disruption of the oleoyl-phosphatidylcholine desaturase *odeA* in *Aspergillus parasiticus* results in diminished growth; delayed germination has been proposed as a countermeasure for controlling this aflatoxigenic species (74). Additionally, volatile organic species emitted by fungi (e.g., (–)-1-octen-3-ol and 10-oxodecanoic acid) play a role in the palatability of mushrooms and may also mediate sporulation and the transition from vegetative to reproductive tissues (10). Separately, targeting Δ^{12} -desaturases, which have no known homologs in humans, in pathogenic basidiomycetes has real potential as selective fungicidal targets. *Cryptococcus neoformans* infections in AIDS and immunosuppressed patients are frequently observed in the clinic; consequently, developing antimicrobial agents targeting *C. neoformans* will markedly improve the health of these patients (55).

Phanerochaete chrysosporium is a widely distributed wood decay homobasidiomycete that has become a model system for studying lignocellulose degradation (41). It harbors an array of peroxidases and degrading lignocellulose as well as aromatic pollutants (14, 26). A role for linoleate (18:2), which may be supplied from endogenous wood lipids or through fungal Δ^{12} -desaturation, in the mediation of lignin degradation has been suggested whereby diffusible lipid-derived peroxy or alkoxy radicals aid in the initial decay of sound wood, particularly in white-rot fungi lacking lignin peroxidase (36, 73). The production of free 18:2 during early colonization of wood meal, followed by extracellular lipid peroxidation and in vitro degradation of nonphenolic lignin, has been shown for the white-rot fungus *Ceriporiopsis subvermispora* (16).

As part of our program to elucidate the biosynthetic networks leading to highly unsaturated natural products in basidiomycetes (e.g., the polyacetylenes) (45), we carried out the cloning and sequence analysis of the gene encoding the sole Δ^{12} -desaturase from *P. chrysosporium*. In this paper, we demonstrate its function through heterologous expression in *Saccharomyces cerevisiae* and show that this enzyme has features distinct from other fungal and plant FAD2 desaturases, which should facilitate future isolation and structure-function analysis of diverged macrofungal desaturases.

MATERIALS AND METHODS

Strains and growth conditions. *P. chrysosporium* (ATCC 24725) used for lipid analysis was grown in modified Norkran's medium amended with dextrose or cellobiose/cellulose (1:4, wt/wt) containing 50 mg of cellulose azure (5 g/liter) at 40°C for 10 days (17). The yeast strain InvSc1 (*MATa his3D1 leu2 trp1-289 ura3-52*) (Invitrogen, Carlsbad, CA) was used in this study. Untransformed yeast strains were cultivated on YPD rich medium (1% Bacto yeast extract, 2% Bacto peptone [Difco Laboratories Inc., Detroit, MI], 2% dextrose) at 30°C with shaking at 250 rpm. Transformed yeast strains were cultured in complete minimal medium with uracil (CM-URA) (2) that was supplemented with dextrose for propagation of clones or 2% galactose (Gal) for expression experiments (at 30°C and 250 rpm). Unusual fatty acids (18:1 Δ^{11} c, 17:1 Δ^{10} c [Nu-Chek Prep, Elysian, MN], and 19:1 Δ^{10} c [Sigma, St. Louis, MO]; 1 mM) were added as ethanol stocks to expression cultures containing 1% Tergitol NP-40 (Sigma). *Escherichia coli*

TABLE 1. Oligonucleotides used as primers for desaturase cloning

Primer name	Sequence (5'–3')
PchryR1	GTGAGCGGCCGAGAGGTATGGCGCGA GGTAGAG
PchryR2	TCAGCGGCCGCAACGAAAACCTGGTCC TGAG
PchryR3	ATAGCGGCCGCTGAGAAAGTGGAGACG ACAG
PchryF1	CACGGGATCCACGCGGCCTGCCAGAGT TCAA
PchryF2	CGTTGGATCCCTCTTTCGCTCCTCCCTTT CCTC
PchryF3	GCGGGATCCTTCTTCCCCATAAAAAATG
PchryF4	CCAGGATCCCATGAAGACCACCGTCAG CAAC
PchryF5	CGAGGATCCCCGTTTCGTTCTGTTTC
PchryF6	TCGCGATCTGGTCGCTTTAC
PchryF7	CGTTCCTCCAGCACACCGA

strain XL1-Blue (Stratagene, La Jolla, CA) was used for DNA cloning and amplification of plasmids.

Cloning and sequencing of the Δ^{12} -desaturase. Chromosomal DNA was isolated from *P. chrysosporium* BKM-F-1767 using previously reported methods (67). mRNA was purified from a 6-day *P. chrysosporium* culture grown on crystalline cellulose (CF-1) (72). Reverse transcription-PCR (RT-PCR) was accomplished using eluted oligo(dT)₂₅ Dynabead-purified mRNA (Dyna, Great Neck, NY) as previously described, except that oligo(dT) was used to prime the RT step (66, 67). A region of the public *P. chrysosporium* version 1.0 genome database that contained a distinctive histidine box motif was initially identified through FAD2 homology searches (41). PCR was performed with the RT product and combinations of forward and reverse oligonucleotide primers designed from predicted exon sequences (Table 1) (Integrated DNA Technology, Coralville, IA) to sequentially amplify intact cDNAs and collectively map the transcriptional locus of the putative *P. chrysosporium* FAD2 gene (PchFAD2). Reaction mixtures contained *Taq* (2 U; New England Biolabs, Beverly, MA) and *Pfu* DNA polymerases (0.4 U; Stratagene), the deoxynucleoside triphosphates (0.2 mM each), and buffer (50 mM Tris-Cl, pH 8.3, 2.5 mM bovine serum albumin, 2% sucrose, 0.1 mM cresol red, 2 mM MgCl₂) with a touchdown thermocycler program (95°C for 30 s denaturation, 66 to 55°C annealing in –0.5°C steps for 30 s, and elongation at 72°C for 90 s, followed by 15 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, and 72°C for 8 min polishing). Overlap PCR used the conditions described above with Vent polymerase and synthetic primers to correct two discrepancies between the existing genomic database and cDNA sequence to the genomic sequence (30).

Construction of yeast expression vectors and cell lines. The full-length PchFAD2 open reading frame (ORF) was amplified (PT 100 Thermocycler; MJ Research, Oldendorf, Germany) with primers PchryF1-F7 and PchryR1-R3 containing BamHI (sense) and NotI (antisense) restriction sites (Table 1). PCR products were purified by Gene Clean (Bio 101, Vista, CA) and digested with BamHI and NotI (Promega, Madison, WI). The resulting PchFAD2 insert was purified and ligated with T4 DNA ligase (Life Technologies-Gibco BRL, Carlsbad, CA) into linearized pYES2 (Stratagene, La Jolla, CA) or pYES2 reengineered with the GAL1 promoter replaced with the *S. cerevisiae* alcohol dehydrogenase (ADH) promoter (21). Constructs were transformed into transformation and storage solution-competent *E. coli* XL-1 Blue by standard techniques (2). Insert-containing plasmids were bidirectionally sequenced to ensure they were mutation free. Plasmids were transformed into *S. cerevisiae* InvSc1 by the lithium acetate-polyethylene glycol method of Gietz (25).

GC-MS analysis of fungal lipids. Methyl esters of cellular lipids from either transformed *S. cerevisiae* or lyophilized *P. chrysosporium* were prepared by incubation in 2% H₂SO₄ in methanol at 80°C for 1 to 2 h. Fatty acid methyl esters (FAMES) were extracted into hexanes, concentrated by evaporation under an N₂ stream, and redissolved in hexanes (100 μ l). For the detection of hydroxy fatty acids, FAMES were derivatized either by heating *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)-trimethylsilyl chloride (99:1, vol/vol) (Sigma, St. Louis, MO) at 70°C for 30 min, concentrating the reaction to dryness under flowing N₂ at 70°C, and dissolving the silylated FAMES in hexanes (100 μ l) or by incubating concentrated FAMES with a 100- μ l solution of BSTFA-acetonitrile-*N,N*-dimethylformamide (2:2:1) for 20 min at room temperature. Fatty acid derivatives were separated and analyzed using an HP5890 gas chromatograph (GC) on a

FIG. 1. (A) Aligned desaturase sequences (ClustalX) with superimposed hydropathy data. The four putative TM domains in PchFAD2 are shown in blue boxes, a hydrophobic domain is marked yellow, and the locations of the His boxes are indicated by pink boxes. (B) Cladogram

30-m DB-23 column (0.25- μ m film thickness; 0.25-mm internal diameter; Supelco, Bellefonte, PA) with an inline Hewlett Packard Model 5972A mass spectrometer (MS) (injector/detector temperatures, 300/280°C; the temperature program consisted of 100°C for 3 min, a rise of 20°C/min to 250°C and a hold for 3 min, and cooling at 20°C/min to 100°C with a 1-min hold; detection parameters consisted of an m/z range of 50 to 350 and 1.52 scans/s). Identification of FAMES was accomplished by the comparison of retention times and parent ions to authentic or literature standards. Pyrrolidine derivatives were prepared by heating FAME samples with glacial acetic acid (25 μ l) and pyrrolidine (250 μ l) at 100°C for 1 h. Cooled samples were diluted with ethyl ether-hexanes (1:1), extracted three times with water, and concentrated under a stream of N_2 . GC-MS samples were separated with a 30-m SP-2380 column (length, 30 m; film thickness, 0.20 μ m; internal diameter, 0.25 mm; Sigma) and analyzed using an HP6890 GC/5975 MS (injector/detector temperatures, 280/250°C; the temperature program consisted of 140°C for 3 min; a rise of 3°C/min to 240°C with a hold for 21.33 min, and cooling at 20°C/min to 140°C; detection parameters consisted of an m/z range of 50 to 140 and 4.14 scans/s). Dimethyl disulfide (DMDS) derivatives of FAMES were prepared using the method of Yamamoto et al. (75), separated by GC-MS using SP-2830 or HP-5-MS columns and the described conditions, and compared to spectra in the literature (60).

Bioinformatics. Multisequence alignments were prepared using ClustalW (70). Phylogenetic analysis was carried out using a ClustalX alignment (70) with PHYLIP, version 3.65 (18). Distances between aligned sequences were determined with PROTDIST using a Dayhoff PAM matrix; branches were positioned using the neighbor-joining method, and the unrooted tree was generated after bootstrapping analysis ($n = 1,000$) with CONSENSE. The tree was drawn using DRAWGRAM, and all branches had >50% support. ConPred II software was used for the prediction of membrane-interacting helices (M. Arai and T. Shimizu, <http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/>).

Nucleotide sequence accession numbers. The cDNA and genomic DNA nucleotide sequences for PchFAD2 have been deposited in GenBank under accession numbers EU852291 and EU939386, respectively.

RESULTS

Isolation of *P. chrysosporium* Δ^{12} -desaturase. The gene corresponding to the sole FAD2 homolog in the *P. chrysosporium* genome, the PchFAD2 allele, was cloned and resequenced. For the purpose of determining the extent of the coding sequence, several sense-strand primers were designed from putative exons that were conserved with *fad2* and surrounding the expected 5' transcriptional start, as well as control primers within expected introns (Table 1; see also Fig. SA1 in the supplemental material and). Antisense primers were designed within and downstream of the largest, highly conserved 3' ORF. A battery of PCRs was performed using the first-strand RT product as a template, prepared from mRNA isolated from a 6-day *P. chrysosporium* culture grown on crystalline cellulose, as well as genomic DNA as a template. Use of the RT template and the primers PchryF5 and PchryR3 (Table 1) led to the successful cloning of a full-length PCR product that contained a single ORF with a stop codon preceding the anticipated *fad2* start codon at the 5' end of the transcript upstream and terminating

the 3' end of the message. *Taq* or *Pfu* polymerase-mediated PCRs with primers that included BamHI and NotI restriction sites allowed the cloning of the amplified DNA into the yeast expression vector, pYES2. Bidirectional sequencing of the cloned cDNA and comparison to the genomic sequence detected two inconsistencies. The *P. chrysosporium* genomic DNA resequenced in our study was found to be identical to the Department of Energy/Joint Genome Initiative genome sequence (see version 2 model at <http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Phchr1&id=125220>); therefore, two PCR-induced errors in our initial cDNA (A435V and R249S) were repaired by overlap PCR.

PchFAD2 gene structure. The PchFAD2 gene was found to bear a number of distinguishing features consistent with the distant relationship of *P. chrysosporium* to plants, Ascomycotina, and Zygomycotina (Fig. 1; see also Fig. SA1 in the supplemental material). Unlike the intron-free ORFs of plant oleate desaturases (7), PchFAD2 contained four introns ranging in length from 53 to 62 nucleotides (nt), with canonical 5'-GTRNGT intron-exon borders and a 5'-CTNAY splice site between 18 to 28 nt from the 3'-YAG splice junctions. Typical for filamentous fungi, a purine (A) occurred the -3 position in advance of the start codon (28). PchFAD2 and an expressed sequence tag (EST) corresponding to a putative *fad2* desaturase from the basidiomycete *Schizophyllum commune* (27) were found to have high G+C contents (61 and 63%, respectively), substantial biases for C in the third position of each codon (55 and 58%, respectively), and very low usage of adenine in the third position (3.6 and 3.1%, respectively) consistent with the codon usage of highly expressed *P. chrysosporium* genes (26, 54). Additionally, two upstream signatures were discernible. The 44-nt sequence between -40 and -83 nt from the observed transcriptional start contains three 5-nt and one 7-nt mirrored repeats that appear to be analogous to the transcriptionally important CT/AG-biased S1 nuclease-sensitive structure from the *Lentinula edodes priA* gene (76). A pyrimidine-rich motif, which is common in highly expressed yeast and *Aspergillus nidulans* genes, was found between -83 to -27 nt (13).

Structural motifs in PchFAD2. The PchFAD2 gene encoded a 443-amino-acid protein that possessed three hallmark histidine box motifs and four putative transmembrane (TM) domains A to D (TM-A to TM-D). This sequence is unusually long for Δ^{12} -desaturases; typical plant Δ^{12} -desaturases (e.g., *Glycine max* FAD2 [GmFAD2] of) are ca. 380 residues in length and bear ca. 36% identity to PchFAD2 (Fig. 1A). Several distinctive regions were identified in PchFAD2: a compact

showing the relationship between plant and fungal Δ^{12} stricto sensu and diverged desaturases (ClustalX). The distance along the branches corresponds to the extent of sequence divergence. Sequences, identified by abbreviations of genus and species (as for the PchFAD2) and experimentally determined activity (ACET, acetylenase; FAD2, Δ^{12} -desaturase; FAD3, Δ^{12} -desaturase; bifunctional enzymes include multiple notations), were obtained from GenBank, unless otherwise indicated, referenced to the listed accession numbers (in parentheses): AnFAD2, *A. nidulans* (AAG36933); CaACET, *C. alpina* (CAA76158); CaFAD2, *Candida albicans* (XP_722258); CcFAD2, *Cryptococcus curvatus* (AAU12575); CciFAD2/3, *Coprinus cinereus* (BAF45335); CnFAD2, *Cryptococcus neoformans* strain b3501 genome annotation corresponding to EST c4h05j2 (XP_570226); CpFAD2, *Crepis palaestina* (CAA76157); GmFAD2, *G. max* FAD2-2 microsomal oleate desaturase isoform (AAB00860.1); HaACET, *Helianthus annuus* (AAO38032); HaFAD2 (AF251843.1); HhACET, *Hedera helix* (AAO38031); KIFAD2, *Kluyveromyces lactis* (CAG98110); KIFAD3 (XP_451551); LeFAD2, *L. edodes* (BAD51484); MaFAD2, *M. alpina* (AF110509); MgFAD2, *Magnaporthe grisea* (XP_365283); MrFAD2, *Mucor rouxii* (AF533361); NcFAD2, *Neurospora crassa* (XP_959528); PcACET, *Petroselinum crispum* (U86374); PchFAD2, (EU852291).

FIG. 2. GC-MS data for PchFAD2 expression. Empty vector (pYES2) and pYES2-PchFAD2 constructs in *S. cerevisiae* InvSc1 cells were expressed for 3 days in CM-URA medium supplemented with 2% Gal at 28°C. FAME analysis of transesterified lipids by GC-MS provided total ion chromatograms in panels A and B. While only saturated and monounsaturated lipids are produced in the absence of PchFAD2 (A), the basidiomycete desaturase causes the accumulation of up to 12% linoleate, substantial 16:2, and diminished levels of monounsaturated fatty acids (B). Panel C shows the mass spectrum for the peak at 10.5 min, which is indistinguishable from an authentic standard of methyl linoleate.

19-residue amino terminus relative to all other oleate desaturases; three insertions relative to the GmFAD2 primary sequence, beginning at PchFAD2 residues 69, 178, and 329; and a 29-amino-acid carboxy-terminal region novel to the basidiomycetes. The extents of the four computationally predicted TM domains, with the bounds for TM-A reconciled with nuclear magnetic resonance characterization of a Δ^{12} -desaturase membrane domains (24), are shown in Fig. 1A. A luminal loop that is isolated from the cytosol-proximate catalytic site begins after P69 and lies between predicted TM helices A and B.

Expression of PchFAD2. We have explored the catalytic properties of PchFAD2 through heterologous expression in *S. cerevisiae* InvSc1 cells. Total FAMES, prepared by methanolysis of whole yeast, were analyzed by GC-MS. In contrast to certain FAD2-type enzymes that we have expressed in our lab (e.g., *Crepis alpina* acetylenase), PchFAD2 demonstrated high levels of activity in yeast up to 32°C (up to 12% of total FAME) (Fig. 2). Maximal PchFAD2 in vivo activity was obtained using Gal-induced expression from the GAL1 promoter in pYES2-derived constructs. Lower diunsaturated fatty acid levels (1 to 3% 18:2 of total FAME) were detected when PchFAD2 was expressed in a construct under the control of the ADH promoter in rich YPD medium (data not shown). In contrast to the C_{16} -rich glycerolipid pool of *S. cerevisiae*, where up to 4% of 16:2 Δ^9 ,12c was observed when PchFAD2 was expressed, cultures of *P. chrysosporium* with dextrose or cellulose carbon sources were found to contain ca. 60% linoleate and 16.3 to 21.5% oleate as the major monounsaturated fatty acid (Table 2). An 18:1/16:1 ratio of 16.0 ± 0.5 was present, irrespective of the carbon source.

Yeast expressing PchFAD2 did not have detectable in vivo hydroxylase activity as examined by trimethylsilylated FAMES (detection level of <0.1% total FAMES). Ricinoleate-containing control samples yielded methyl 12-trimethylsilyloxyoleate under two derivatization methods.

Three supplementary fatty acids, 17:1 Δ^{10} c, 18:1 Δ^{11} c, and 19:1 Δ^{10} c, were added to PchFAD2 expression cultures to examine the regiospecificity of PchFAD2. Each of the fatty acids was incorporated by the yeast although no additional effect was noted for the 11-*cis* fatty acid. For the 10-*cis* substrates, 6.3% of the 19:1 supplement was converted to a new desaturated

lipid. FAMES from this culture were derivatized with DMDS to allow the location of the double bonds. For the DMDS derivative, one and two peaks were resolved by GC using HP-5 and SP-2380 columns, respectively. Mass spectra from peaks at 37.4 and 37.8 min contained fragmentation patterns consistent with published spectra for 13,14- and 10,11-bis(methylthio) adducts of 19:2 $\Delta^{10,13}$ (data not shown) (60). Additionally, the pyrrolidine derivative of the 19:2 FAME was analogous to the published pyrrolidide of 18:2 $\Delta^{10,13}$ (W. W. Christie, www.lipidlibrary.co.uk/ms/arch_pyr/pyr2/py0479.htm). Unexpectedly, the C_{17} substrate blocked all Δ^{12} -desaturation activity.

Effect of temperature. The plant FAD2 homologs display temperature-sensitive activity when heterologously expressed in yeast. For example, linoleate levels accumulated by *Arabidopsis thaliana* FAD2-expressing *S. cerevisiae* decreased from 9.2% at 15°C to 0.9% at 28°C (11). Remarkably, the temperature dependence of PchFAD2 is opposite that of the plant enzymes, ranging from $0.25\% \pm 0.03\%$ at 18°C to $3.3\% \pm 1.0\%$ at 32°C when expressed with the ADH promoter in YPD medium (data not shown). The same trend is apparent for the pYES2 constructs in CM-URA medium with Gal (Table 3). Considerable 16:2 accumulation (up to 4% of total FAME) was observed with a temperature dependence similar to 18:2.

TABLE 2. Effect of carbon source on the total fatty acid composition of *P. chrysosporium* cultured in Norkran's broth

Acyl chain	% Total fatty acids by carbon source of culture ^a	
	Cellulose	Dextrose
16:0	16.2 $\pm$ 4.0	12.0 $\pm$ 1.6
16:1	0.99 $\pm$ 0.03	1.38 $\pm$ 0.39
18:0	1.94 $\pm$ 0.93	1.20 $\pm$ 0.08
18:1	16.3 $\pm$ 0.7	21.5 $\pm$ 3.7
18:2	57.8 $\pm$ 7.3	60.2 $\pm$ 2.8
20:0	0.92 $\pm$ 0.46	0.20 $\pm$ 0.20
22:0	TR	TR
24:0	2.81 $\pm$ 1.2	1.92 $\pm$ 1.50
26:0	1.31 $\pm$ 0.37	0.77 $\pm$ 0.50
28:0	1.64 $\pm$ 0.46	0.87 $\pm$ 0.64

^a Detected by GC-MS analysis of FAMES. TR, trace.

TABLE 3. Effect of temperature on yeast total fatty acid composition in the heterologous expression of PchFAD2 determined by GC-MS analysis of FAMES

Temp (°C)	Acyl lipid composition (% of total FAMES) ^a					
	16:0	16:1	16:2 Δ 9,12	18:0	18:1	18:2 Δ 9,12
15	11.2 $\pm$ 1.3	62.1 $\pm$ 2.5	0.98 $\pm$ 0.11	3.1 $\pm$ 0.2	14.5 $\pm$ 0.3	8.1 $\pm$ 0.6
22	25.0 $\pm$ 1.2	51.5 $\pm$ 1.6	1.6 $\pm$ 0.1	4.2 $\pm$ 0.3	10.1 $\pm$ 0.2	7.7 $\pm$ 0.8
30	25.5 $\pm$ 0.8	42.5 $\pm$ 1.6	4.1 $\pm$ 0.2	7.0 $\pm$ 0.3	8.9 $\pm$ 0.05	12.1 $\pm$ 0.5

^a As determined on InvSc1 cells cultured in CM-URA medium with 2% Gal for 3 days.

While plant desaturases do produce 16:2, most FAD2 enzymes exhibit high chain length discrimination. For PchFAD2 expressed in *S. cerevisiae*, the 18:2/16:2 product ratio decreases from 10.6 to 4.8 over 4 days, potentially due to a shift in in vivo monounsaturated substrate concentrations.

DISCUSSION

In this paper, we describe the cloning and characterization of the oleate desaturase from *P. chrysosporium*. The genomic sequence of PchFAD2 correlates to the region of nt 340170 to 341724 of scaffold 20 in the *P. chrysosporium* version 2.0 genome and contains four introns. This is a distinct gene structure from FAD2 plant desaturase homologs that may be valuable for evolutionary analysis. PchFAD2 is the sole oleate desaturase homolog in *P. chrysosporium*, giving it the unique catalytic ability to control PUFA levels. The presence of a putative acyl-coenzyme A dehydrogenase gene immediately adjacent to PchFAD2 (see the Department of Energy/Joint Genome Initiative genome sequence version 2 model at <http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Phchr1&id=135142>) has potential significance in controlling lipid composition.

Protein attributes. The primary structure of PchFAD2 is anchored by two pairs of putative TM domains which locate two His box domains on the cytosolic membrane face and a final His box, which is positionally conserved in the carboxyl terminus of the protein. For the three His box motifs of membrane-bound desaturases, the eight histidines that have been shown by Shanklin et al. to be functionally required are conserved, and the relative positioning of the TM domains is similar in each of the fungal desaturases (Fig. 1A) (62). In agreement with the observations of Arondel et al. for ω -3 desaturases (1), no classical carboxy-terminal K(H)DEL ER retention signal was found, nor could KSKIN and YNNKL ER retrieval motifs be located in PchFAD2 (43). We detected a conserved motif including amino acids 398 to 413, (R/K)XCK F(V/I)EXXXD(V/I)(V/A)F(Y/F)KN, that is found in all fungal desaturases (Fig. 1A). While the function of this domain remains to be validated, the similarity of this domain with zygomycete FAD2s, such as that from *Mortierella alpina*, suggests that the aromatic motif F(Y/F)KN may control ER retention. Variation between the NH₂- and COOH-terminal regions of basidiomycete FAD2s may mediate organism-specific targeting or signaling.

Plant and fungal *sensu stricto* Δ^{12} -desaturases are known to function with the electron transport components cytochrome *b*₅:NADH oxidoreductase and cytochrome *b*₅ from yeast (61). The substantial activity of PchFAD2 and other Δ^{12} -desaturases with C₁₆ substrates in *S. cerevisiae* may arise serendipitously

from the unusually high levels of 16:1, which is a less common fatty acid in basidiomycetes. The apparent desaturation efficiency $[(18:2)/(18:2 + 18:1)]/[(16:2)/(16:2 + 16:1)]$, as measured by the percentage of total FAMES provides an initial measure the preference for C₁₈ substrate over the C₁₆ chain length. For PchFAD2, the apparent desaturation efficiency of 6.61 demonstrates a large preference for oleate; by extension from measured 16:1 levels in cellulose- and dextrose-containing media, 16:2 would be expected at levels of 0.13 and 0.17% of the total cellular lipids, respectively. The activity of PchFAD2 stands in contrast to the general rejection of 16:1 as a substrate by yeast-expressed FAD2s from the phylum *Zygomycota* (52, 59). It is possible that, through catabolic editing, *P. chrysosporium* eliminates 16:2 from the acyl lipid pool or that the substrate selectivity is altered by membrane lipid composition. The conversion of 16:1 Δ 9c to 16:2 Δ 9c,12c by *Trichoderma* spp. is strongly suppressed when 18:2 and 18:3 are present (64). The high levels of 18:2 from cultured *P. chrysosporium* were comparable to data in the literature (68), and although 18:3 was absent, *P. chrysosporium* may act similarly.

Desaturases are known that possess regioselectivity in which the location of dehydrogenation correlates to distances from the carboxyl terminus (Δ^n -), methyl end (ω -x), or a preexisting alkene ($\nu+3$) (60, 65) although this property is often annotated without verification. As the regioselectivity of Δ^{12} - and $\nu+3$ desaturases is masked by 16:1 Δ 9c and 18:1 Δ 9c substrates, we examined the positional preference through the use of the atypical substrates, 17:1 Δ 10c, 18:1 Δ 11c, and 19:1 Δ 10c. When provided at a high concentration (1 mM), 19:1 Δ 10c was desaturated to 19:2 Δ 10c,14c, as verified by the analysis of the fatty acyl pyrrolidide fragmentation pattern and the DMDS adducts (60). Under these conditions, 16:2 Δ 9c,12c and 18:2 Δ 9c,12c from endogenous Δ^9 - and PchFAD2-catalyzed Δ^{12} -desaturation were formed at nearly unfed levels. The 18:1 Δ 11c isomer was not accepted by PchFAD2, and, unexpectedly, 17:1 Δ 10c blocked desaturation completely. Therefore, PchFAD2 functions as a $\nu+3$ desaturase that preferentially acts on *cis*-9-unsaturated substrates.

Microbial enzymes capable of both Δ^{12} - and ω -3 bifunctional dehydrogenation activities are identifiable as a desaturase subfamily (12). In the current study, we note that overall sequence similarity primarily sets the basidiomycete desaturases apart from other fungal and plant enzymes, while differences in underlying regioselectivity in each cluster may result from a smaller subset of residues (Fig. 1B). In FAD2/FAD3 (microsomal ω -3) enzymes from ascomycetes, residues corresponding to T101, V105, L133, V159, and I296 have been found through mutagenesis to be Δ^{12} -desaturase-promoting residues (31, 34, 44). Each of the corresponding residues is present in PchFAD2

supporting the 18:1-desaturating activity in basidiomycetes shown through our expression studies. By their respective 74% and 54 to 62% amino acid similarities to PchFAD2, *S. commune* (BG739693) and *Pleurotus ostreatus* (AT004257.1 and AT004124.1) EST sequences are likely FAD2 homologs. *S. commune* and *Fistulina hepatica* are very closely related species (47), a fact that is particularly relevant as we will use *F. hepatica* in a screen for diverged FAD2 acetylenases.

Many plant FAD2 desaturases possess an underlying 12-hydroxylation activity (4). The similarity of hydroxylation and desaturation-competent active sites can be seen through a site-directed mutational analysis in which the substitution of all seven residues in an plant oleate desaturase for the corresponding residues in plant hydroxylases resulted in a substantial enhancement of hydroxylation activity (4, 5). Reciprocal experiments from the same study showed that substitution of the four residues in a hydroxylase augmented desaturase activity and, later, that two more residues modulate hydroxylation/desaturation ratios (4). The absence of hydroxylase activity in PchFAD2 is remarkable. Consequently, PchFAD2 provides a null background for the identification of counterparts to plant hydroxylation markers in the fungal desaturases.

The plant FAD2 homologs have temperature-sensitive activity when heterologously expressed in yeast. For example, linoleate levels accumulated by *A. thaliana* FAD2-expressing *S. cerevisiae* decreased from 9.2% at 15°C to 0.9% at 28°C (11) and mirrors the low-temperature PUFA accumulation generally observed in plants. The corollary was effectively demonstrated when tobacco showed improved acclimatization to elevated temperatures when the chloroplast membrane ω -3 desaturase *FAD7* was silenced (49). Remarkably, the temperature dependence of PchFAD2 is opposite that of the plant enzymes irrespective of the ADH or GAL1 promoters. The temperature dependence of in vivo fatty acid accumulation may derive from posttranscriptional changes in mRNA, post-translational protein modification or trafficking, or temperature effects on the inherent catalytic efficiency of PchFAD2. In the case of the basidiomycete *L. edodes* FAD2, mycelia transcripts remained essentially constant following a temperature downshift while in vivo activity appeared to increase at lower temperatures during yeast expression and increased dramatically during fruiting body development (57). For two isoforms of soybean oleate desaturase, FAD2-1A and FAD2-1B, transcript levels were found to vary minimally, but ubiquitinylation and proteasome degradation appeared to correlate with protein levels; catalytic activity could be diminished by phosphorylation of a proteolysis-relevant serine (69). No high-probability Ser/Thr kinase or protein/Thr kinase sites were found in PchFAD2. Amino acids in the NH₂-terminal domain (residues 1 to 44) and in the later third of the FAD2-1B (residues 241 to 334) were identified as potential determinants of thermal stability, with the interaction of multiple domains likely required (69).

P. chrysosporium is a model mesophilic fungus with an optimum growth temperature of 39°C (6). As poikilotherms, fungi respond to temperature stress by adjusting the unsaturation of cellular lipids to control membrane fluidity. Transduction of temperature effects may be mediated by changes in transcription or at the protein level that are dependent on specific signaling pathways. The finding in this work of in-

creasing in vivo desaturase activity at elevated culture temperatures observed for PchFAD2 expression in yeast is unique to plant and fungal Δ^{12} -desaturases. As heterologous expression of FAD2-like genes is not believed to significantly alter the framework of endogenous lipid processing enzymes in yeast, we interpret the expression results to indicate that PchFAD2 has higher thermal stability or higher kinetic parameters in the elevated temperature regime than other FAD2 enzymes.

As our in vivo activity pattern is reversed, we surmise that the increased activity at elevated temperatures may reside in the activity profile for the enzyme and correlate with the high optimal growth temperature of *P. chrysosporium* (40°C). Thermophilic fungi are known to contain higher levels of 18:1 and saturated fatty acids and to preferentially incorporate saturated fatty acids in their polar lipids (48). For the assayed Δ^{12} -desaturase system of the yeast *Lipomyces starkeyi*, specific activity was maximal at 40°C although the enzyme was found to denature rapidly at higher temperatures (40). PUFAs have been found to suppress the activity of FAD2 enzymes (22) and cause changes in membrane fluidity that could affect enzyme activity or substrate availability (19), both of which provide alternate hypotheses for the atypical behavior of PchFAD2. Ultimately, optimal temperature for PchFAD2 will be determined by in vitro assay.

Conclusion. The lack of crystallographic information for the microsomal desaturases is attributable to their notorious resistance to purification (23, 33). The potential that the thermal stability of PchFAD2 will exceed that of the plant enzymes and its novel structural motifs suggest that PchFAD2 may embody an enzyme sufficiently distinct from the plant desaturases to facilitate structural definition in the microsomal desaturase family. Future studies will examine the origin of the anomalous temperature sensitivity of PchFAD2 compared to plant enzymes and may facilitate the exploration of the role of desaturases in wood degradation.

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