Acetylenic specialized metabolites containing one or more carbon-carbon triple bonds are widespread, being found in fungi, vascular and lower plants, marine sponges and algae, and insects. Plants, moss, and most recently, insects, have been shown to employ an energetically difficult, sequential dehydrogenation mechanism for acetylenic bond formation. Here, we describe the cloning and heterologous expression in yeast of a linoleoyl 12-desaturase (acetylenase) and a bifunctional desaturase/acetylenase. Functional diversity in fungal fatty acid synthesis reveals two cooperative multifunctional FAD2 enzymes that provide cis-enzyme and trans-alkene building blocks of the naturally occurring acetylenes.

The on-line version of this article (available at http://www.jbc.org) contains supplemental data, Tables S1 and S2, and Figs. S1–S4.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) HM036206 and HM036207.

The first acetylenase from the Pacific golden chanterelle, Cantharellus formosus

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In an effort to obtain the 5′ ends of two Cantharellus cibarius PFAD2 homologs identified by shotgun sequencing (0745 and 0807), the phage library was screened with DNA probes (~600 base pairs) prepared by PCR-amplification (Vent DNA polymerase; New England Biolabs) from excised phagemids with primers specific for the FAD2 homologs (supplemental Table S1). The probes were radiolabeled with dCTP* (PerkinElmer Life Sciences; 185 MBq, 50 mCi) by a random-primed reaction using the Ladderman labeling kit (TaKaRa, Shiga, Japan). Plaque lifts, hybridization, and screening were carried out by standard methods (29, 30). Positive phage clones from a two-step screen were recovered, and phagemids were excised with ExAssist helper phage in the SOLR E. coli strain (Stratagene). Excised plasmids were sequenced by in-house automated DNA sequencing (Dynemic ET Terminator Cycle Sequencing kit; Amersham Biosciences) with a T7 promoter primer and compared with the initially sequenced EST clones. A cDNA that was slightly shorter than the original 0807 shotgun sequenced phagemid was obtained from this screen. Comparison of the sequences with each other and other diverged desaturases indicated that the initial phagemid clone was full-length, which was later confirmed by expression in yeast. Despite a second round of library screening for 0745 using a probe amplified with primers closer to the 5′ end of the initial EST clone (0745a@100, 0745a@600), we were unable to obtain the entire 5′ end of the gene.

A 5′ rapid amplification of cDNA ends (5′-RACE) approach was used to obtain the remainder of the 0745 PFAD2 homolog from poly(A)^+ RNA isolated from C. formosus fruiting bodies with the BD SMARTRACE cDNA amplification kit (BD Biosciences/Clontech). First-strand cDNA synthesis was accomplished with a poly(dT) primer and BD Power Script reverse transcriptase (BD Biosciences/Clontech), the C. formosus 0745 desaturase gene was amplified from the 5′-RACE-ready cDNA using a universal primer mix and a gene-specific primer (0745a@190), and the product was isolated by agarose gel electrophoresis gel extraction (BD Biosciences NucleoTrap Gel Extraction kit). 5′-RACE products were then subcloned into pGEM-T by T/A cloning (Promega pGEM-T Easy Vector System II) and transformed into ultra-competent JM109 E. coli (Promega), and insert-containing plasmids were sequenced as described. All oligonucleotide primers used are shown in supplemental Table S1.

Construction of Yeast Expression Vectors and Cell Lines—The full-length 0745 and 0807 open reading frames were amplified with primers containing BamHI (sense) and EcoRI (antisense) restriction sites for insertion into yeast expression vectors (supplemental Table S1). PCR products and DNA fragments were purified by Gene Clean (Bio 101), and purified, digested BamHI/EcoRI (Promega) C. formosus desaturase inserts were ligated into pYES2 or pESC-His (Stratagene) with or without an N-terminal His_GlySer fusion tag with T4 DNA ligase (Invitrogen) and transformed into competent XL-1 Blue cells by standard techniques (30). Sequence-validated plasmids were transformed into competent InvSc1 by a standard lithium acetate method (31). Strains for co-expression were transformed simultaneously with dual selection.

Acetylenic Lipid Biosynthesis in a Basidiomycete

FIGURE 1. Biosynthesis of polyacetylenic metabolites in Basidiomycetes. The metabolic progression is supported by the feeding studies leading to highly unsaturated, bioactive metabolites, exemplified by compounds 1-4. Biotransformations functionally characterized in this paper produce lipids accumulated by (bold) or absent from (dashed) C. formosus.
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Desaturase Expression and GC/MS Analysis of Fungal Lipids—Heterologous expression of *C. formosus*-diverged desaturase genes was induced by cultivation of transformed *Saccharomyces cerevisiae* in 5 ml of CM—ura or CM—ura—hehis with galactose (2 g/100 ml) at the indicated temperatures with shaking at 250 rpm. Fatty acid methyl esters (FAMEs) of yeast lipids were prepared and analyzed by gas chromatography-mass spectrometry (GC-MS, HP 5890-HP 5972 MS; DB-23 column) as previously described (28) and identified by comparison of retention time and parent ions to authentic standards. Cells supplemented with exogenous fatty acids were washed twice with 10 mg/ml Nonidet P-40 and once with H2O to remove fatty acids not incorporated into cellular lipids. Dimethylxoxazolines were prepared according to the method of Christie (32). Synthesis and characterization of trans fatty acid authentic standards are provided in the supplemental data.

FAME stereoisomers were additionally separated with an HP 5890 gas chromatograph using a SP-2380 capillary column (Aldrich, 30 m × 0.25 mm). The temperature program was 150 °C for 10 min, ramped to 180 °C at 2 °C/min, then ramped at 20 °C/min to 250 °C and cooled by 20 °C/min back to the initial temperature. The injector and detector were set to 250 °C, and the gas flow rate was 0.9 ml/min. Dimethylxoxazoline derivatives were separated using the temperature program 100 °C or 3 min, ramped 5 °C/min to 250 °C, 250 °C for 3 min, and cooled 50 °C/min to 100 °C; inlet and detector temperatures, 250 °C.

**RESULTS**

Cloning of *C. formosus* Diverged Desaturases—We initiated our search for diverged desaturases in the *C. formosus* recently distinguished from *C. cibarius* as a unique species (33), genome through a cDNA sequencing approach. Phagemids (1037) were sequenced, and the resulting sequences were screened using a BLAST algorithm for homologs of known plant and fungal desaturases allowing us to identify two putative diverged Δ^{12} desaturases, Cf0745 and Cf0807 (5 representatives), and a Δ^{9} desaturase, Cf0820. The initial sequences were used in hybridization screening of the cDNA phage library for full-length clones. Of 1.5 × 10^{9} plaque-forming units screened, 22 were identified as positives for Cf0807. A secondary screen resulted in 3 positive clones; however, a 5′-RACE strategy was necessary to obtain the 5′ end of the Cf0745 cDNA, affording a predicted 1389-bp FAD2 homolog cDNA encoding a 462-amino acid protein (accession number HM036207). Full-length nucleotide and amino acid sequences for both Cf0807 and Cf0745 are found in supplemental Fig. S2 and have been submitted to GenBank™.

**Predicted Protein Structure Analysis of Cf0745 and Cf0807**—An unrooted phylogenetic tree demonstrated the relationships of the chanterelle proteins to recognized desaturases and was suggestive of biochemical function (Fig. 2). Cf0745 (CfDES) was found to segregate with fungal proteins known only to carry out the prototypical oleate desaturase reaction. Cf0807 (CfACET), however, was not imbedded deeply within the mapped fungal clades but was, rather, lying on a discrete spur.

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**FIGURE 2. Cladogram relating the chanterelle enzymes to representative FAD2, FAD3, and bifunctional desaturases.** The graph shows the phylogenetic relationships between non-heme dirron enzymes diverged in chemo- and regioselectivity, assembled by a neighbor-joining algorithm from boot-strapped (n = 100) sequence alignments, plotted using DRAWGRAM (Phylip 3.65) (51). Families of desaturases and hydroxylases are bracketed by normal-weight lines, acyltetrolases by bold lines, enzymes with activity regioselective to the Δ15/ω3 position by the dotted line, and enzymes with multiple locations of reactivity by the dashed line. Chemoselectivity of the associated desaturases are: DES, Δ^{12} desaturase; Δ^{12}/Δ^{15}, bifunctional Δ^{12}/Δ^{15} desaturase; ACET, Δ^{17} acyltetrolase; CONJ, Δ^{12} conjugase; ω-3, ω-3 desaturase; ω-6, microsomal ω-6 desaturase; EPOX, 12 epoxygenase; 12-OH, 12-hydroxylase; TDES, trans-Δ^{12} desaturase; Δ^{15}, Δ^{15} limoleoyl desaturase. The GenBank™ accession numbers for the displayed sequences are: AnDES, AAG369933; AtD15 (ER), P48623; Atw-3 chloroplast, P46310; CoDES, XP_722258; CalpACET, CAAs67158; CoDES, AAU12575; CoDES, NP_502560; Cw-3, NP_001023560; CoDES, A4W42920; CoDES, A4G42259; CoCONJ, A4G42260; CpDES, CAAs67157; CpePOX, CAAs67156; Cw-3, BAB78717; DsCONJ, AA572901; DsTDES, AA572902; FgDES, EAA75859; FgD12/D15, ABB88516; FmDES, AB898015; FmD12/D15, ABB88516; GmDES1, AA08859; GmDES2, AA008650; GmDES15 (ER), P48625; HaACET, AA038082; HaDES, AAL69892; HhACET, AA038031; IIDES, XP_455420; LdD15, AA578690; LeDES, BAD51484; Lt120H, AAC32575; MaDES, A881754; McDES, BB69056; MgDES, XP_365283; MgD12/D15, XP_366936; MfDES, AAM97924; NcDES, XP_955528; NcD12/D15, XP_328856; PcaCET, AAB08697; PdCDES, ACL13289; PtDES, AAO23564; Rf120H, AAC49910; SynDES, NP_896789; VfCONJ, AAAN87574; Vid15, AAC98967. An, Aspergillus nidulans; At, Arabidopsis thaliana; Ca, Candida albicans SC5314; Calp, *C. alpina*; Cc, Cryptococcus curvatus; Ce, Caenorhabditis elegans; Ch, Cryptococcus neoformans var. neoformans JEC21; Co, Calendula officinalis; Cp, Crepis palustre; Cv, Chlorella vulgaris; Da, Dimorphotheca sinuata; Fg, Gibberella zeae PH-1; Fm, Fusarium moniliforme; Gm, Gymnax max; Ha, Helianthus annuus; Hh, Hedera helix; Ik, Kluyveromyces lactis; Ld, Limnanthes douglasii; Le, Lentinula edodes; Lf, Lesquerella fendleri; Ma, Mortierella alpina; Mc, Mucor rouxii; Nc, Neurospora crassa OR74A; Pc, Petroselinum crispum; Pch, Phanerochaete chrysosporium; Rhizopus oryzae PT, Phaeodactylum tricornutum; Rf, Ricinus communis; RoDES; Syn, Synechococcus sp. WH8102; Vf, Vernicia fordii.

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Local sequence alignments showed several distinguishing features between the plant and fungal kingdoms. Histidine box motifs that are believed to bind two active-site iron atoms and are critical to enzyme function (34, 35) are present and are positionally conserved in translations of both Cf0745 (CfDES) and Cf0807 (CfACET) (Fig. 3). The i-1 position of motif I is near the predicted ER retention sequences (36–38). Histidine box II is found near the predicted cytosolic face of the ER bilayer.

Expression of C. formosus Diverged Desaturases—We have explored the catalytic properties of the C. formosus predicted diverged desaturases through heterologous expression in S. cerevisiae. Our experience with Basidiomycete diverged desaturases indicates that a N-terminal hexahistidine tag results in increased levels of in vivo desaturation activity when compared with the untagged protein (28). We expressed both the untagged and the His$_6$-tagged predicted diverged desaturases cloned from C. formosus and determined the temperature dependence of desaturase-product accumulation. Acid-catalyzed methanolation of whole yeast preparations produced FAMEs that were analyzed by GC-MS. Expression of each untagged Cf0745 and Cf0807 and the His$_6$ fusion proteins H$_6$Cf0745 and H$_6$Cf0807 resulted in the production of new fatty acids not present in the empty vector control (Table 1 and supplemental Table S2, A and B). Expression of H$_6$CfDES and H$_6$CfACET increased the doubling time compared with the empty vector control by 13 and 34%, respectively, at 15 °C. However, the fusion proteins resulted in significantly higher product accumulation (up to 4-fold greater for the dominant acyl products) than their untagged counterparts. Therefore, to more easily follow in vivo activity of Cf0745 and Cf0807, we conducted subsequent heterologous expression studies with the His$_6$ fusion proteins.

Total ion chromatograms and mass spectra of the new fatty acids produced by Cf0745 and Cf0807 were consistent with 16:2 \(\Delta^9,12\) and 18:2 \(\Delta^9,12\), demonstrating that both of the predicted enzymes have \(\Delta^9\) desaturation activity and are functionally related to the FAD2 family of desaturases, as suggested by the gene and protein alignments. H$_6$Cf0745 was an extremely active desaturase with a preference for the C$_{18}$ substrate producing almost 20% of the total fatty acids as 18:2 \(\Delta^9,12\) and more than 7% 16:2 \(\Delta^9,12\) at 15 °C. Close inspection of the retention times of the C$_{16}$- and C$_{18}$-diunsaturated products of H$_6$Cf0807 (Fig. 4A) suggested that they were not identical to the H$_6$Cf0745 products. Rather, the 16:2 and 18:2 products of H$_6$Cf0807 were demonstrated to have exclusively the trans double bond configuration at the

**Figure 3.** Local sequence alignments comparing the three iron-binding His box domains in FAD2-divergent desaturases from plants and fungi. The first His residue in each box is labeled $i$, and features are indexed negatively amino-proximate and positively to the carboxyl side of $i$. The sequence abbreviations are described in the Fig. 2 legend except: CaACET (=CalpACET), CAA76158 (14); AtDES, AAA32782 (1); DcACET, ACE73033 (15); DcFCET, AAO38036 (15); RoDES, AAV52631.

**Table 1**

| Temperature | Construct | 16:0 | 16:1 | 16:2 | 18:0 | 18:1 | 18:2 |
|-------------|-----------|------|------|------|------|------|------|
| °C          |           |      |      |      |      |      |      |
| 15          | pYES2     | 12.2 ± 0.9 | 57.7 ± 1.8 | 0 | 3.51 ± 0.20 | 26.6 ± 0.8 | 0 |
| 22          | 19.8 ± 0.5 | 52.9 ± 1.4 | 0 | 3.22 ± 0.14 | 24.0 ± 0.8 | 0 |
| 30          | 20.6 ± 0.8 | 44.3 ± 4.1 | 0 | 7.26 ± 1.90 | 27.9 ± 1.4 | 0 |
| 15          | H$_6$Cf0745$^a$ | 13.8 ± 1.5 | 45.9 ± 1.3 | 7.57 ± 0.73 | 4.32 ± 0.15 | 9.58 ± 0.2 | 18.9 ± 1.7 |
| 22          | 21.2 ± 0.9 | 49.4 ± 1.5 | 3.31 ± 0.40 | 4.01 ± 0.26 | 10.6 ± 0.5 | 11.4 ± 0.9 |
| 30          | 18.0 ± 1.0 | 49.0 ± 1.3 | 0.61 ± 0.29 | 5.37 ± 0.06 | 25.0 ± 1.0 | 1.90 ± 1.99 |
| 15          | H$_6$Cf0807$^b$ | 11.8 ± 0.3 | 59.6 ± 0.6 | 3.02 ± 0.21 | 2.74 ± 0.16 | 21.9 ± 0.2 | 0.90 ± 0.07 |
| 22          | 19.9 ± 0.9 | 53.8 ± 2.0 | 0.64 ± 0.03 | 2.75 ± 0.29 | 22.6 ± 0.8 | 0.30 ± 0.04 |
| 30          | 17.6 ± 1.0 | 51.3 ± 0.6 | 0 | 4.56 ± 0.23 | 26.5 ± 0.6 | 0 |

$^a$ For diunsaturated lipids, \(\Delta^9,12\).

$^b$ For diunsaturated lipids, \(\Delta^9,12\).
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C12 position by separation of the stereoisomers with a specialized SP-2380 capillary GC column and co-elution of the H6Cf0807 16:2 and 18:2 products with authentic Δ9c,12t FAMEs prepared in our laboratory (data not shown). H6Cf0807 demonstrated a preference for the C16 substrate producing ~3% 16:2 Δ9c,12t and 0.9% 18:2 Δ9c,12t at 15 °C (Table 1).

Growth of the expression cultures at 15, 22, and 30 °C resulted in dramatic differences in the level of diunsaturated fatty acids for both H6Cf0745 and H6Cf0807 fusions (Table 1) and the untagged proteins (supplemental Table S2) with each showing substantially more activity at lower temperatures. H6Cf0745 desaturation products made up more than 26% of the total FAMEs at 15 °C compared with 2.5% at 30 °C. Similarly, H6Cf0807 products were almost 4% of the total FAMEs at 15 °C and were not detectable at 30 °C (Table 1). This temperature dependence is consistent with plant FAD2 homologs when heterologously expressed in yeast (39); however, it is opposite that of another fungal desaturase that we recently described (PchFAD2) that demonstrated higher activity at 30 °C compared with 15 °C (28).

To produce the accumulated natural product dehydrocrepenenic acid, chanterelle must have both FAD2/3 desaturase and acetylenase activities. The yeast expression of H6Cf0745 and H6Cf0807 establishes that H6Cf0745 is a classical FAD2 desaturase. The production of the trans Δ12 double bond by H6Cf0807 suggested that this enzyme is an acetylenase, as this activity has also been observed in the C. alpina acetylenase CREP1 (40). To test for acetylenase activity in the C. formosus FAD2 homologs, we supplemented yeast expressing H6Cf0745 or H6Cf0807 with 18:2 Δ9c,12c and analyzed FAMEs. In all cell lines the supplemented 18:2Δ9,12 composed >75% of the total FAMEs (Table 2). H6Cf0745 expression resulted in the production of only 16:2 Δ9,12 and 18:2 Δ9,12 (indicated by increased levels above the empty vector control) as seen in the non-supplemented cultures. However, expression of H6Cf0807 resulted in the production of crepenenic acid (18a,1Δ9,12c) to >0.5% of the total FAMEs as determined by comparison of retention time and mass spectra with dimethyloxazoline and FAME authentic standards and literature data (Figs. 4B and supplemental Fig. S3) (15, 32). No C16 acetylenic fatty acid was detected. Supplementation with 18:2 Δ9c,12a, α-linolenic acid (18:3 Δ9,12,15), or γ-linolenic acid (18:3 Δ6,9,12) did not result in the production of new fatty acids (data not shown). Crepenenate supplementation of cultures at low concentrations

FIGURE 4. GC-MS analysis of the novel acyl modifications resulting from the recombinant chanterelle desaturase and acetylenase. FAME total ion chromatograms collected for total acyl lipids of Cf0807 are expressed in yeast at 15 °C in the absence (A) and presence (B) of 0.5-μl exogenous linoleic acid.

TABLE 2
Substrate specificity of C. formosus diverged desaturases

| Constructs | 16:0 | 16:1 | 16:2Δ9,12 | 18:0 | 18:1 | 18:2Δ9,12 | 18:1Δ9c,12a | 18:2Δ9c,12a,14c |
|------------|------|------|-----------|------|------|-----------|-------------|----------------|
| pYES2      | 13.6 ± 3.1 | 4.00 ± 0.12 | ND | 2.47 ± 0.62 | 2.06 ± 0.16 | 77.9 ± 3.8 | ND | ND |
| H6Cf0745   | 10.3 ± 0.1 | 2.52 ± 0.17 | 0.40 ± 0.02 | 1.81 ± 0.06 | 0.63 ± 0.05 | 84.4 ± 0.2a | ND | ND |
| H6Cf0807   | 12.8 ± 1.4 | 3.75 ± 0.40 | 0.17 ± 0.03 | 2.43 ± 0.38 | 2.11 ± 0.27 | 78.2 ± 2.5  | 0.52 ± 0.08 | ND |

| Constructs | 16:0 | 16:1 | 16:2Δ9,12 | 18:0 | 18:1 | 18:2Δ9,12 | 18:1Δ9c,12a | 18:2Δ9c,12a,14c |
|------------|------|------|-----------|------|------|-----------|-------------|----------------|
| pYES2      | 17.0 ± 0.7 | 21.3 ± 1.5 | ND | 4.98 ± 0.10 | 18.1 ± 1.2 | ND | 38.6 ± 3.3 | ND |
| vH6Cf0745  | 21.1 ± 0.6 | 20.6 ± 1.3 | 1.48 ± 0.29 | 5.75 ± 0.70 | 7.7 ± 1.0 | 8.20 ± 0.30 | 31.9 ± 1.4 | 3.26 ± 0.19 |
| H6Cf0807   | 21.6 ± 0.2 | 21.7 ± 3.6 | 0.96 ± 0.26 | 6.45 ± 0.66 | 15.0 ± 2.0 | 0.43 ± 0.19 | 33.9 ± 4.9 | ND |
| PchFAD2    | 34.8 ± 1.3 | 18.2 ± 1.4 | 1.40 ± 0.22 | 10.7 ± 0.5 | 7.99 ± 0.48 | 4.68 ± 0.15 | 18.7 ± 3.6 | 3.46 ± 0.26 |

* Fatty acid supplemented to expression culture.
* Δ9c,12a.
* Δ9c,12c.
* Δ9c,12t.
* Δ9c,12c.
* Δ9c,12t.

Expression temperature of 30 °C.
to avoid its toxicity resulted in the production of another new fatty acid upon heterologous expression of \( H_c \)Cf0745 (>3.2% of total FAMEs; Table 2). This fatty acid was determined by mass spectrometry of authentic dimethyloxazoline and FAME standards to be dehydrocrepenynic acid (supplemental Fig. S3) (15).

Metabolic channeling in dehydrocrepenynate biosynthesis between the desaturase and acetylenase enzymes was demonstrated by the co-expression of Cf0745 and Cf0807 in \( S. \) cerevisiae. When \( H_c \)Cf0745 and \( H_c \)Cf0807 were co-expressed from independent plasmids, the production of 18:2 \( \Delta^{9c,12c} \), 18:1 \( \Delta^{9c,12a} \), and 18:2 \( \Delta^{9c,12a,14c} \) from endogenous oleic acid was observed (Fig. 5). Channeling through the biosynthetic pathway from oleic to dehydrocrepenynic acid was more efficient than when each Cf0745 and Cf0807 were supplied substrates through media supplementation as demonstrated by the % conversion of 18:2 \( \Delta^{9c,12c} \) into 18:1 \( \Delta^{9c,12a} \) (0.67% for supplementation versus 1.51% conversion of enzymatically produced 18:2 \( \Delta^{9c,12c} \)) and the subsequent conversion of 18:1 \( \Delta^{9c,12a} \) to 18:2 \( \Delta^{9c,12a,14c} \) (20.9% versus 43%, respectively) (Table 3). The \( \Delta^{12c} \) desaturation efficiency ratio for \( C_{16} \) versus \( C_{18} \) substrates, and acetylenation/trans-desaturation ratio were 3.43 and 1.37, respectively, upon co-expression.

**DISCUSSION**

In this report we describe the cloning and functional characterization of two diverged desaturases from the Basidiomycete \( C. \) formosus and show that the production of dehydrocrepenynic acid from oleic acid in fungi may be accomplished by the activity of just two FAD2-like activities.

Simple acetylenic fatty acids are known from a small number of plants and fungi, but unfortunately, yeast expression of plant FAD2 acetylenases and epoxidases, a straightforward system for functional characterization of both plant and fungal desaturases, often results in little or no detectable activity (14, 15). A similar result was observed during the current studies of Cf0807; however, His\(_5\) fusion-tagging serendipitously amplified the heterologous acetylenase activity and allowed us to redesignate the gene CfACET.

A number of basidiomycotal \( \Delta^{12} \) desaturases have been characterized by our group and others (28, 41–43). In this study, Cf0745, an enzyme similar to known fungal desaturases, showed high desaturation activity for both \( C_{16} \) and \( C_{18} \) substrates and was consequently renamed CfDES. Although fungal desaturases have been observed not to be transcriptionally regulated by unsaturated fatty acid levels, decreasing temperature normally boosts activity (43). The temperature dependence of heterologously expressed CfDES and CfACET activity was similar to plants but dissimilar to the Phanerochaete FAD2 (28) and is consistent with ectomycorrhizal \( C. \) formosus thriving in a temperate plant-like environment.

Although the expected spacing and organization of the three His box domains for a fungal \( \Delta^{12} \) or \( \Delta^{15} \) -3 desaturase is present, a pairwise comparison with CfACET revealed a maximum sequence identity of 48% to fungal \( \Delta^{12} \) desaturases and a meager 25% to plant FAD2-like acetylenases. Local analysis of the histidine box motifs established clear attributes of both plant acetylenases and fungal desaturases in CfACET. Acetylenases may have diverged from a single ancient \( \Delta^{12} \) or \( \omega-3 \) desaturase paralog. Subsequent functional diversification (44) resulted in relatively homogeneous subfamilies of the fungal FAD2, plant acetylenases, and mixed regioselectivity desaturases; CfACET appears to be the first member of a new acetyl enase subfamily. Given the large evolutionary distance between plants and fungi, resolving whether the ability of fungi to produce polyacetylenes is a gain or loss of function phenotype requires substantially more data.

The multifunctional nature of the diverged \( \Delta^{12} \) desaturases demonstrated by both of the \( C. \) formosus enzymes is apparently of broad occurrence. Although the details of the hydrogen abstraction are sparse due to the difficulty in gaining structural and spectroscopic data into these integral membrane proteins, oxygen activation by a reduced Fe(II)-Fe(II) core and stepwise fission of the carboxyl-proximate C-H bond followed by che moselective loss of the second hydrogen atom is the generally held model for both \( C=\equiv C \) and \( C=\equiv C \) bonds (45–47). Nevertheless, the origins of selectivity leading to acetylenes and desaturases are not currently distinguishable de rigueur by comparisons at the primary sequence level, and residues promoting acetylenase activity have yet to be located.

![FIGURE 5. Total ion chromatogram of yeast total FAMES demonstrating desaturase-acetylenase co-expression.](image)

**TABLE 3**

Coexpression of \( C. \) formosus diverged desaturases

| Constructs          | Fatty acids |          |          |          |          |          |          |          |
|---------------------|------------|----------|----------|----------|----------|----------|----------|----------|
|                      | 16:0       | 16:1     | 16:2 \( \Delta^{9c,12c} \) | 16:2 \( \Delta^{9c,12a} \) | 18:0     | 18:1     | 18:2 \( \Delta^{9c,12c} \) | 18:1 \( \Delta^{9c,12a} \) | 18:2 \( \Delta^{9c,12a,14c} \) |
| pYES2/pESc          | 18.3 ± 1.4 | 43.0 ± 2.4 | ND       | ND       | 4.00 ± 0.29 | 34.8 ± 2.3 | ND       | ND       | ND       |
| \( H_c \)Cf0745/ \( H_c \)Cf0807 | 20.1 ± 1.0 | 32.0 ± 1.4 | 3.56 ± 0.13 | 7.87 ± 1.3 | 6.1 ± 0.28 | 11.0 ± 0.78 | 18.9 ± 0.87 | 0.29 ± 0.06 | 0.22 ± 0.07 |

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**Acetylenic Lipid Biosynthesis in a Basidiomycete**

The observation that trans- and acetylenic fatty acid production is intertwined in the *Cantharellus* enzyme deserves comment. The 9c,12t products of CfACET trans-desaturase activity are apparent metabolic deadends as the trans double bond appears to prevent the correct positioning for hydrogen abstraction leading to the acetylenic unit (16, 40). The absence of 18:2 Δ9c,12t in chanterelle lipids is consistent with relatively low oleate concentrations in the mushroom. In contrast, *C. alpina* accumulates 3.3% 18:2 C. alpina low oleate concentrations in the mushroom. In contrast, 12-acetylenase CREP1 (40). Striking is the stereochemical continuum of the desaturase activities from purely 18:2 Δ9c,12t-producing FAD2s through the cis/trans mixtures of CREP1 and noted in insect pheromone biosynthesis and plant sphingolipid Δ8 desaturation (48) to pure 18:2 Δ9c,12t in CfACET. Efficient streaming in chanterelle of oleate through linoleate to the acetylenase, selectivity of lipid storage pathway enzymes, or modifications to the structure or environment of CfACET in fungal membranes may suppress in vivo trans-desaturation.

Although in the absence of high resolution structural data, a precise structural determinant of trans-desaturation cannot be unequivocally defined, this activity does seem to be an inherent property of the Δ12-acetylenes and other diverged FAD2 enzymes. Stymne and co-workers (40) showed that 18:2Δ9c,12c is produced by abstraction of the pro-(R) hydrogens at C12 and C13, whereas 18:2Δ9c,12t is produced by scission of the (12R,13S) hydrogens; similar selectivity of prochiral hydrogens has been noted for Δ8-sphinolipid desaturases (48). Diverged FAD2 desaturases exhibit a primary kinetic isotope effect of 4–15 at C12 and near unity at C13 revealing sequential C-H abstraction (45, 49). The short lifetime anticipated for the putative allylic radical at C12 makes improbable a 180° cis-eclipsed to anti-staggered rotation before excision of the activated hydrogen at C13. Although evidence points to a transversely flexible binding cleft in the microsomal desaturases rather than the constrained tunnel of the soluble desaturases (50), to avoid electrophilic attack of activated oxygen on the π-system, acetylenase binding pockets must precisely position the rigid linoleate substate while templating oleate in a range of conformations capable of producing one or both alkene stereochimistries ([supplemental Fig. S4](#)). The combined data suggest that stereochemical control in desaturation and acetylenases may be better described by a window model rather than the cis-desaturase eclipsed binding paradigm (Fig. 6). In a 12-trans-yielding eclipsed transoid conformation, the presentation of the reactive hydrogens to the activated oxygen is expected to only deviate slightly from linoleate. A less restrictive binding pocket could allow a small rotation to a torsionally strained cisoid conformation and permit the formation of an E/Z mixture. Normal Δ12 desaturases may have substrate channels or pockets within the active site fully capitated toward a cis-eclipsed conformation. In this model, CfACET has a less flexible active site than CREP1. Variations in diverged desaturase activity may additionally lie in the precise positioning of the iron atoms or perturbations of the protein shells surrounding the active site.

Expression of plant acetylenases in somatic soybean embryos resulted in the accumulation of crepenyenic and dehydrocrepenyenic acids (15); however, the metabolic origin of the conjugated Δ14 double bond was not clear at the time. A possibility was that crepenyenic acid had an altered substrate conformation in the active site of an endogenous ω-3 Δ15 desaturase (FAD3), resulting in the desaturation at the Δ14 position. Our studies argue against this, as *S. cerevisiae* does not have FAD3 activity. Additionally, we found that a Myc-tagged ω-3 desaturase from tung was not active with crepenyenic. Another possibility was that the acetylenases were able to introduce a triple bond into both 18:2 Δ9,12 as well as 18:3 Δ9,12,15, both of which are present in high levels in soybean. For 18:3 Δ9,12,15, this would occur with concomitant migration of the (15Z)-double bond to the 14-position. We found that CfACET is inactive with α-linolenic acid (18:3 Δ9,12,15), an observation that could not be easily made in the soybean system and is strong evidence against this possibility. Here we showed that only when *S. cerevisiae* cells were supplemented with crepenyenic acid and expressing Cides or co-expressing Cides and CfACET was dehydrocrepenyenic acid produced. This clearly demonstrates that Δ14-desaturating activity lies within the catalytic abilities of the canonical Cides and may result from altered substrate geometries in the FAD2 active site. Furthermore, we observed the general ability to form a (Z)-enyne with PchFAD2 (Table 2) and the oleate desaturases from Euphorbia and soybean, which are not known to produce acetylenic compounds. Together, and bolstered by the observation that the Tpi-PGFAD enzyme responsible for the production of a moth sex pheromone is capable of successive Δ11-desaturation, Δ15-acetylenation, and Δ15-desaturation reactions to produce 16:2 Δ11,15 (17), these results are consistent with a generally open active site in the Δ12- but not ω-3 desaturases. A shortened acetylenic chain sets up a r+2 desaturation reaction despite the limited conformational possibilities for C11–C14 in 18:1Δ9c,12a. This facility to use a collection of acyl substrates to produce multiple products implies a flexible binding pocket, a hallmark of secondary metabolic processes.

As the pathway to dehydrocrepenyenic acid was reconstituted in our *S. cerevisiae* expression system, the compositional importance of this lipid in the chanterelle, and the fact that no other FAD2 diverged desaturase was found in our shotgun sequencing, Cides and CfACET fulfill all of the FAD2-like activities required to produce dehydrocrepenyenic acid in fungi and demonstrate that the two FAD2 homologs are necessary and sufficient for the production of dehydrocrepenyenic acid.

**FIGURE 6.** **Consolidated binding pocket model for cis- and trans-desaturases and acetylenases.** To accommodate the region-specific production of (12E)-alkenes in all of the methyl-proximal acetylenases, the alkyl chain at C12-C13 is reorganized within a 90°–120° dihedral angle window. Within these bounds, the projection of C13 normal to the linoleate-defined plane for a cis-desaturation is minimized, whereas the vinyl C-H bond orientation is constrained. Torsion about the C12-C13 bond coordinated with H13 abstraction is postulated to lead to cis- and trans-alkenes.
from oleic acid. Additionally, because the conversion of enzymatically derived intermediates of the three-step pathway in the co-expression experiments was more efficient than conversion of supplemented substrates, it appears that the flux of desaturation and acetylenylation products produced in situ in the ER is more directed than that of exogenously supplied fatty acids. Many complexities of polyacetylenic natural product biosynthesis remain to be resolved; however, the current work clearly indicates that the number of required diverged desaturases may be lower than the number of dehydrogenation steps due to strong multifunctional activities.

In conclusion, enzymatic participants necessary for the initial three polyunsaturation reactions in C. formosus were discovered that give underlying molecular support for the crepennate pathway. Significant variation between fungal and plant acetylenases and desaturases provide a wealth of diversity in dehydrogenative lipid specialized metabolism. Future correlation of entrained structure/function relationships will yield an improved microsomal desaturase structural model aiding in the engineering of bio-based lipid products.

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