Caenorhabditis elegans Δ12-Desaturase FAT-2 Is a Bifunctional Desaturase Able to Desaturate a Diverse Range of Fatty Acid Substrates at the Δ12 and Δ15 Positions[s]

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Background: *Caenorhabditis elegans* CeFAT-2 was previously characterized as a fatty acid Δ12-desaturase.
Results: CeFAT-2 can introduce second double bond in a range of fatty acid substrates that have a Δ9 double bond.
Conclusion: CeFAT-2 is a bifunctional Δ12/Δ15-desaturase.
Significance: This is the first evidence that the nematode Δ12-desaturase is a bifunctional desaturase that can produce highly unusual desaturated fatty acids.

Caenorhabditis elegans FAT-2 has been characterized as fatty acid Δ12-desaturase able to desaturate C16 and C18 fatty acids. However, in this report we show that when expressed in yeast cells this enzyme can also catalyze Δ15 desaturation. This results in the production of both linoleic acid ($\omega 6$ C18:2$^{\Delta 9,12}$) and linolenic acid ($\omega 3$ C18:3$^{\Delta 9,12,15}$) from oleic acid (C18:1$^{\Delta 9}$) substrate, and hexadecadienoic acid ($\omega 4$ C16:2$^{\Delta 9,12}$) and hexadecatrienoic acid ($\omega 1$ C16:3$^{\Delta 9,12,15}$) from palmitoleic acid (C16:1$^{\Delta 9}$) substrate. In addition, this enzyme can also produce C14:2$^{\Delta 9,12}$, C15:2$^{\Delta 9,12}$, C17:2$^{\Delta 9,12}$, and C18:4$^{\Delta 6,9,12,15}$ when C14:1$^{\Delta 9}$, C15:1$^{\Delta 9}$, C17:1$^{\Delta 9}$, and C18:3$^{\Delta 6,9,12}$ substrates are available in yeast cells. Mass spectrometry analysis of 2,4-dimethyloxazoline modification of fatty acid methyl esters confirms the positions of all newly formed double bonds. These results indicate that when expressed in yeast the *C. elegans* Δ12-desaturase CeFAT-2 shows a characteristic of a bifunctional Δ12/Δ15-desaturase and has a great deal of elasticity with respect to fatty acid chain length in being able to accept fatty acids ranging from C14 to C18. Interestingly, despite possessing a bifunctional Δ12/Δ15 desaturation activity, phylogenetic analysis suggests that *C. elegans* Δ12-desaturase CeFAT-2 might have arisen independently from other reported dual Δ12/Δ15-desaturases from fungi and protozoa.

In recent years, fatty acid synthesis pathways in many species have been genetically dissected, leading to the identification of fatty acid desaturation and chain elongation genes. For example, the *Caenorhabditis elegans* polyunsaturated fatty acid synthesis pathway has been shown to contain at least seven desaturases and two elongases (1, 2). These include Δ9-desaturases (FAT-5, FAT-6, FAT-7), Δ12-desaturase (FAT-2), Δ15-desaturase or $\omega 3$-desaturase (FAT-1), Δ5-desaturase (FAT-4), and Δ6-desaturase (FAT-3). Among the desaturases, FAT-2 was earlier confirmed as a Δ12-desaturase acting on oleic acid (C18:1$^{\Delta 9}$) to pro-

duce linoleic acid (LA,[2] $\omega 6$, C18:2$^{\Delta 9,12}$) (3) whereas FAT-1 was shown to be a Δ15-desaturase acting on $\omega 6$ fatty acid LA, for example, to form α-linolenic acid ($\omega 3$, C18:3$^{\Delta 9,12,15}$) (4–6).

Fatty acid desaturases possess at least five specificities: chemoselectivity (desaturation *versus* hydroxylation), regioselectivity (double bond position), stereoselectivity (cis *versus* trans), chain length specificity, and acyl carrier specificity (acyl-CoA *versus* acyl-lipid substrate). Historically, three main types of regioselectivities have been distinguished (6). Desaturases with Δx regioselectivity introduce a first double bond x carbon atoms away from the carboxyl end. A second group comprises ωy-desaturases, and these insert a double bond at position y referenced from the methyl end (for example, ω3-desaturase). Finally, there are the v+z-desaturases which require a preexisting double bond as reference point (v) and catalyze the formation of a new double bond z carbon atoms further along the acyl chain, either toward methyl end (*i.e.* Δ12-desaturase) or toward carboxyl end (*i.e.* front end Δ4- or Δ5-desaturase). These modes of regioselectivity are not mutually exclusive as illustrated by several bifunctional fungal Δ12/ω3-desaturases. For example, in addition to the Δ12-fatty acid desaturase members, separate bifunctional Δ12/ω3-fatty acid desaturases have been identified from fungi *Fusarium moniliforme* (7) and *Aspergillus nidulans* (8). These bifunctional enzymes desaturate oleic acid C18:1$^{\Delta 9}$ at Δ12 position to form C18:2$^{\Delta 9,12}$ and can also carry out a further desaturation at the Δ15 position to form ω3-fatty acid C18:3$^{\Delta 9,12,15}$. Additionally, these fungal desaturases also convert $\omega 6$ substrates, including LA, γ-linolenic acid (C18:3$^{\Delta 6,9,12}$), dihomo-γ-linolenic acid (C20:3$^{\Delta 8,11,14}$), and arachidonic acid (C20:4$^{\Delta 5,8,11,14}$) to their ω3 products and show an obvious preference for the C18 substrates, especially LA (7, 8). A bifunctional Δ12/Δ15-desaturase that directs the synthesis of highly unusual hexadecatrienoic acid (C16:3$^{\Delta 6,9,12}$, $\omega 1$) has also been isolated from the free-living soil protozoon *Acanthamoeba cas-*

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[2] The abbreviations used are: LA, linoleic acid; AtFAD2, *Arabidopsis* Δ12-desaturase; CeFAT-1, *C. elegans* fatty acid desaturase 1; CeFAT-2, *C. elegans* fatty acid desaturase 2; DMOX, 2,4-dimethyloxazoline; FAME, fatty acid methyl ester.

tellanii (9) and phytopathogenic fungus, *Claviceps purpurea* (10).

Here, we report the identification of a novel undescribed catalytic function for the previously characterized *C. elegans* $\Delta12$-desaturase CeFAT-2 enzyme. When expressed in yeast cells, CeFAT-2 displays characteristics of a bifunctional $\Delta12/\Delta15$-desaturase and has a great deal of elasticity with respect to fatty acid chain length in being able to desaturate fatty acids ranging from C14 to C18. Finally, phylogenetic analysis suggests that despite possessing a bifunctional $\Delta12/\Delta15$ desaturation activity, CeFAT-2 appears to have arisen independently from other reported dual $\Delta12/\Delta15$-desaturases from fungi (7, 8) and protozoa (9).

EXPERIMENTAL PROCEDURES

Fatty Acids and Fatty Acid Methyl Ester (FAME)—Free fatty acids and FAME standards C8–C24 were purchased from Sigma. C18:1$^{\Delta12}$ was chemically synthesized. Other fatty acid substrates were purchased from Sigma or Cayman Chemical Company.

Gene Subcloning—The *C. elegans fat-2* gene (GenBank accession number AF240777) was PCR-amplified from cDNA with restriction sites HindIII and XhoI at the ends of PCR primers (forward, 5′-GTAAAGCTTGGCCCCCGACG and reverse, 5′-TGTCTCGAGATTTTGTAGTTTATTG) and cloned in yeast expression vector pYES2 (Invitrogen) with a *Gal1* promoter to generate pXZP278. As a control, the *Arabidopsis* $\Delta12$-desaturase gene *AtFAD2* (At3g12120) coding sequence was also PCR-amplified with primers (forward, 5′-TCCGTCGA-CATGGGTGCAGGTGGAAGA and reverse, 5′-CGGGG-TACCTCATAACTTATTGTTGTACCAG). The PCR product was cloned into pGEM-T Easy (Promega) and subcloned into pYES2 as EcoRI blunt-ended SpeI fragment to generate plasmid pXZP279. The gene sequences in these plasmids were confirmed by sequencing the entire amplified regions. The *C. elegans fat-1* gene (GenBank accession number L41807) was synthesized by GeneArt and cloned into yeast expression vector with a *Gal1* promoter as an EcoRI-XhoI fragment in which restriction sites were built in before and after the start and stop codons, generating plasmid pXZP586.

Expression of Genes in Yeast and Gas Chromatography Analysis—Plasmids were introduced into yeast strain S288C (11) cells by heat shock as described previously (12). Yeast feeding with different exogenous fatty acids was essentially as described previously (12). FAMEs were analyzed with a Agilent N6890 GC on a 60-m BPX70 column (0.25-mm inner diameter, 0.25-μm film thickness, SGE), or Thermo Polaris Q GC-MS on a 30-m BPX70 column. The column temperature was programmed as an initial temperature at 170 °C holding for 1 min, ramping to 200 °C at 5 °C/min and 210 °C at 2.5 °C/min then to 240 °C at 10 °C/min, and finally holding for 3 min on Agilent GC, or 60 °C holding for 1 min, ramping to 120 °C at 9 °C/min then to 250 °C at 50 °C/min, and holding for 1 min on a Thermo GC-MS. Helium was the carrier gas. Identification of peaks was based on comparison of retention time with standard FAME and confirmed with MS acquired and processed with Xcalibur™ software from Thermo Electron Corporation.

Double bond positions in the FAME were confirmed with 2,4-dimethyloxazoline (DMOX) adducts by derivatizing the
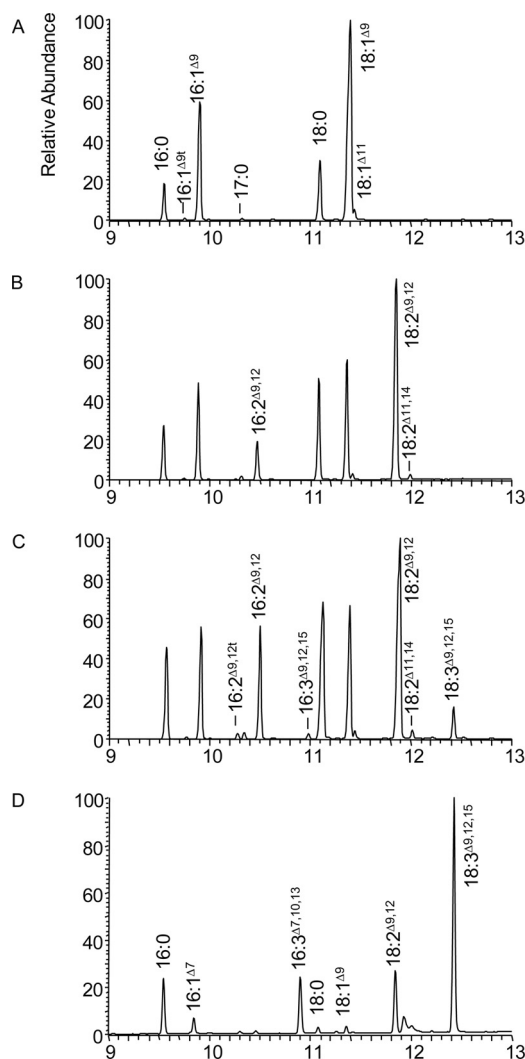

FIGURE 1. **Partial gas chromatogram of FAMEs from yeast cells or *Arabidopsis* leaf.** *A–C*, yeast cells expressing vector pYES2 only, AtFAD2, and CeFAT-2. *D*, *Arabidopsis* leaf.

FAMEs with 2-amino-2-methyl-propanol at 190 °C overnight, cooling, and acidifying with 0.1 N HCl before extracting with dichloromethane (13), and analyzed as described previously for FAMEs (12).

RESULTS

C. elegans $\Delta12$-Desaturase CeFAT-2 Also Carries Out $\Delta15$ Desaturation—In this study, CeFAT-2 is expressed under the control of an inducible *Gal1* promoter in *Saccharomyces cerevisiae* strain S288C (*MATa, his3-d200 leu2-d1 trp-1-d63 lys2-d202 ura3-52*). In the absence of any exogenous fatty acid feeding, it produces two new peaks in addition to the new but expected C16:2$^{\Delta9,12}$ or C18:2$^{\Delta9,12}$ (Fig. 1). As expected, the main fatty acids in yeast cells carrying empty vector are C16:0, C16:1$^{\Delta9}$, C18:0, and C18:1$^{\Delta9}$ (Fig. 1*A*). Trace amounts of C16:1$^{\Delta9trans}$, C17:0, and C18:1$^{\Delta11}$ are also found. The two abundant new peaks present in the FAMEs of yeast cells expressing both *Arabidopsis* AtFAD2 and nematode CeFAT-2 (both enzymes were previously demonstrated as $\Delta12$-desaturases) are confirmed as hexadecadienoic acid (C16:2$^{\Delta9,12}$) and LA (C18:2$^{\Delta9,12}$), as shown in Fig. 1, *B* and *C*. In addition, trace amounts of
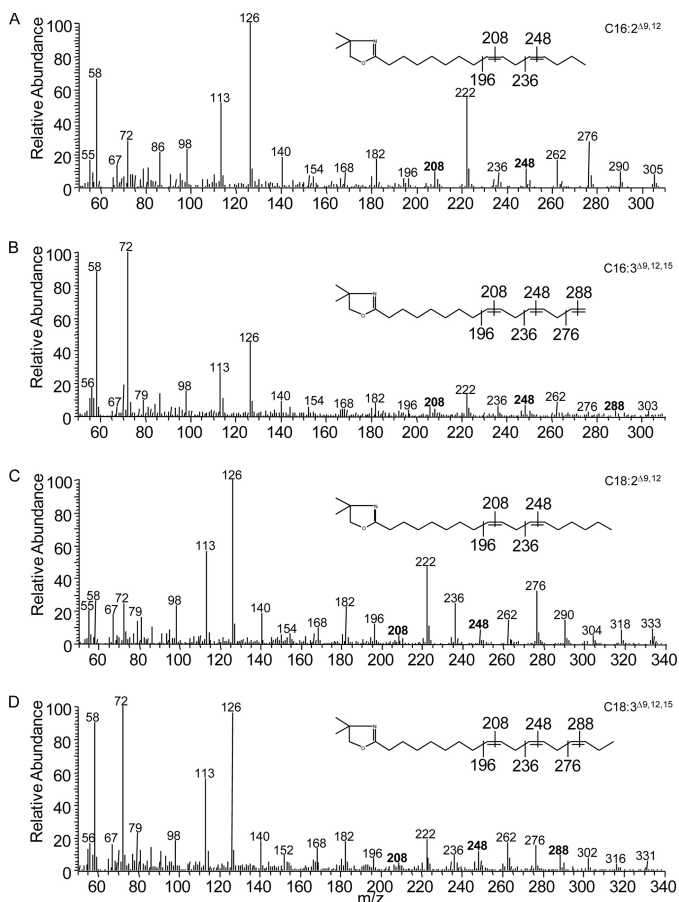
FIGURE 2. **Mass spectrometry of DMOX derivatives of major CeFAT-2 products.** *A–D*, DMOX adducts of C16:2$^{\Delta 9,12}$, C16:3$^{\Delta 9,12,15}$, C18:2$^{\Delta 9,12}$, and C18:3$^{\Delta 9,12,15}$, respectively.

Fatty acid	Vector	AtFAD2	CeFAT-2
C16:0	6.55	8.68	9.64
C16:1$^{\Delta 9}$	25.60	15.38	11.57
C16:1$^{\Delta 11}$	<0.01	<0.01	<0.01
C16:2$^{\Delta 9,12}$	0.00	6.62	10.39
C16:3$^{\Delta 9,12,15}$	0.00	0.00	0.46
C18:0	10.59	14.88	18.34
C18:1$^{\Delta 9}$	55.60	16.97	13.17
C18:1$^{\Delta 11}$	1.02	0.46	0.47
C18:2$^{\Delta 9,12}$	0.00	35.30	31.79
C18:2$^{\Delta 11,14}$	0.00	0.42	0.62
C18:3$^{\Delta 9,12,15}$	0.00	0.00	3.26
Δ12 Desaturation on C16:1 (%)	0.0	30.1	47.3
Δ12 Desaturation on C18:1 (%)	0.0	67.5	72.7
Δ15 Desaturation on C16:2 (%)	0.0	0.0	4.3
Δ15 Desaturation on C18:2 (%)	0.0	0.0	9.3

function as a Δ15-desaturase and produce a ω3 C18:3$^{\Delta 9,12,15}$ product from C18:1$^{\Delta 9}$ as well as a highly unusual ω1 C16:3$^{\Delta 9,12,15}$ fatty acid from C16:1$^{\Delta 9}$. It is noteworthy that Δ12 desaturation activity of CeFAT-2 is 8–10 times higher than its Δ15-desaturase activity (Table 1). Finally, other tiny peaks can also be observed in FAMEs from yeast expressing CeFAT-2. These are confirmed either as trans-isomers of corresponding cis-unsaturated fatty acids (data not shown) or as possible elongation products produced by yeast cells (see below).

Divergent Fatty Acid Substrates Desaturated by CeFAT-2— Further extensive yeast feeding with exogenous fatty acids shows that CeFAT-2 can desaturate C14:1$^{\Delta 9}$, C15:1$^{\Delta 9}$, and C17:1$^{\Delta 9}$ at the Δ12 position to produce C14:2$^{\Delta 9,12}$, C15:2$^{\Delta 9,12}$, and C17:2$^{\Delta 9,12}$. Fig. 3 shows the GC analysis of yeast FAMEs expressing vector only, AtFAD2, and CeFAT-2. When fed with C14:1$^{\Delta 9}$, in addition to the C16:2 and C18:2 products, a new peak with mass spectra of C14:2 can be found only in cells expressing either of the Δ12-desaturases (Fig. 3 and supplemental Fig. S2). The new products are confirmed by DMOX adducts of the FAMEs as C14:2$^{\Delta 9,12}$, C16:2$^{\Delta 9,12}$, and C18:2$^{\Delta 9,12}$ (supplemental Fig. S2 and data not shown). In addition, low amounts of C16:3 and C18:3 are also detected in FAMEs of yeast expressing CeFAT-2 but not in yeast expressing AtFAD2, again confirming the additional Δ15 desaturation activity of CeFAT-2. Also, in cells expressing both AtFAD2 and CeFAT-2, trace amounts of additional dienoic peaks are detected eluting after the C16:2$^{\Delta 9,12}$ and C18:2$^{\Delta 9,12}$ peaks. These peaks display the typical mass spectra of C16:2 and C18:2 (234, 266 *m/z* and 262, 294 *m/z*, respectively). These are confirmed by DMOX adducts of the FAMEs to be C16:2$^{\Delta 11,14}$, C18:2$^{\Delta 11,14}$, possibly elongated from C14:2$^{\Delta 9,12}$ and C16:2$^{\Delta 9,12}$. When provided with C14:1$^{\Delta 9}$, yeast cells can elongate this fatty acid to C16:1$^{\Delta 11}$ substantially, as shown by the new peak eluting after C16:1$^{\Delta 9}$ in FAMEs of all yeast samples (Fig. 3). Trace amounts of C18:1$^{\Delta 11}$ and C18:1$^{\Delta 13}$ are also detected, presumably the elongation products of C16:1$^{\Delta 9}$ and C16:1$^{\Delta 11}$, respectively. The mass spectra of DMOX adducts C16:1$^{\Delta 11}$, C16:2$^{\Delta 11,14}$, C18:1$^{\Delta 11}$, C18:1$^{\Delta 13}$, and C18:2$^{\Delta 11,14}$ are shown in supplemental Fig. S2.

When fed with C15:0, yeast cells can elongate part of this fatty acid into C17:0, as shown in FAMEs of yeast cells expressing either pYES2 vector only or Δ12-desaturases (see supplemental Fig. S3). Both C15:0 and C17:0 are desaturated into

C18:2$^{\Delta 11,14}$, presumably elongated from C16:2$^{\Delta 9,12}$, were also identified. Interestingly, additional new peaks in the FAMEs of yeast expressing CeFAT-2 are also observed (Fig. 1*C*). One of them has the same retention time as the C18:3$^{\Delta 9,12,15}$ standard and the dominant fatty acid present in *Arabidopsis* leaf (Fig. 1*D*). The other new peak appeared before C18:0 in Fig. 1*C* and has a retention time comparable with C16:3$^{\Delta 7,10,13}$, an abundant *Arabidopsis* leaf fatty acid (Fig. 1*D*). The identities of these new peaks are confirmed by MS analysis (see supplemental Fig. S1). The mass spectra matched to the mass spectra of standards C16:2 (234, 266 *m/z*), C16:3 (232, 264 *m/z*), C18:2 (262, 294 *m/z*) and C18:3 (260, 292 *m/z*).

The positions of the double bonds in the new products are confirmed as C16:2$^{\Delta 9,12}$, C16:3$^{\Delta 9,12,15}$, C18:2$^{\Delta 9,12}$, and C18:3$^{\Delta 9,12,15}$ by MS analysis of DMOX adducts of the FAMEs, as shown in Fig. 2. Fig. 2*A* shows the structure of C16:2 (molecular ion of *m/z* 305), with the double bond present in positions Δ9 and Δ12 located by the gap of 12 atomic mass units between *m/z* 196 and 208, 236 and 248, respectively. Fig. 2*B* shows the structure of C16:3 with molecular ion of *m/z* 303 and indicates that this C16 fatty acid contains three double bonds. These double bonds are confirmed to be present at positions 9, 12, and 15 and located by the gap of 12 atomic mass units between *m/z* 196 and 208, 236 and 248, 276 and 288, respectively. Similar structures of DMOX adducts of C18:2 and C18:3 are shown in Fig. 2, *C* and *D*. These results suggest that CeFAT-2 can also
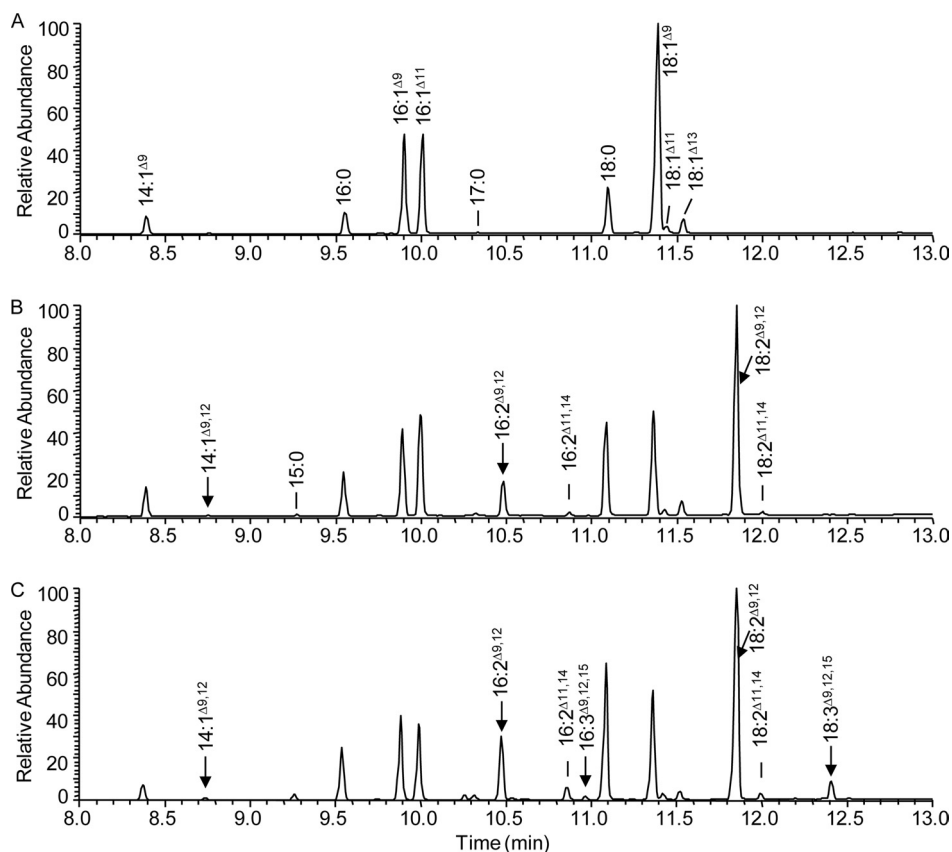
FIGURE 3. **Partial gas chromatogram of yeast feeding for CeFAT-2 with C14:1$^{\Delta 9}$.** *A–C*, FAMEs of yeast S288C expressing pYES2 vector only, AtFAD2, and CeFAT-2, respectively, fed with C14:1$^{\Delta 9}$. New desaturated products from AtFAD2 or CeFAT-2 are indicated by *arrows*.

monounsaturated fatty acid C15:1 and C17:1, by the host cell $\Delta 9$-desaturase, as these peaks are also present in cells expressing vector only. These peaks are confirmed as C15:1$^{\Delta 9}$ and C17:1$^{\Delta 9}$ by DMOX adducts rather than C15:1$^{\Delta 10}$ and C17:1$^{\Delta 10}$ (see supplemental Fig. S3). There is another C17:1 peak eluting after C17:1$^{\Delta 9}$ in all three samples, presumably C17:1$^{\Delta 11}$, an elongation product of C15:1$^{\Delta 9}$. Compared with FAMEs from yeast cells expressing vector only, both AtFAD2 and CeFAT-2 produce several new peaks, including C15:2$^{\Delta 9,12}$ (co-eluting with trace amounts of trans-C16:1$^{\Delta 9}$), C16:2$^{\Delta 9,12}$, C17:2$^{\Delta 9,12}$, and C18:2$^{\Delta 9,12}$. In addition, trace amounts of C17:2$^{\Delta 11,14}$ and C18:2$^{\Delta 11,14}$ are also detectable in yeast cells expressing both AtFAD2 and CeFAT-2, presumably elongated from the desaturation product C15:2$^{\Delta 9,12}$ and C16:2$^{\Delta 9,12}$, respectively. Again, cells expressing CeFAT-2 also produce C16:3$^{\Delta 9,12,15}$ and C18:3$^{\Delta 9,12,15}$, evidence of $\Delta 15$ desaturation activity of CeFAT-2. Because C17:2$^{\Delta 9,12}$ is only produced in low amounts, we cannot confirm whether this product can be further desaturated to C17:3$^{\Delta 9,12,15}$ by CeFAT-2.

When fed with C18:3$^{\Delta 6,9,12}$, there is no detectable product produced from the fed substrate in yeast cells expressing vector only (Fig. 4*A*) or AtFAD2 (Fig. 4*B*), although the cells incorporate an abundant amount of the substrate. In contrast, CeFAT-2-expressing cells produce C18:4$^{\Delta 6,9,12,15}$ via a desaturation at the $\Delta 15$ position (Fig. 4*C*). The characteristic ion for the *n*-3 structure, at *m/z* 107/108 (14), was apparent (see supplemental Fig. S4) as well as the ion for $\Delta 6,9$ double bonds at *m/z* 194. The double bond positions are also confirmed at
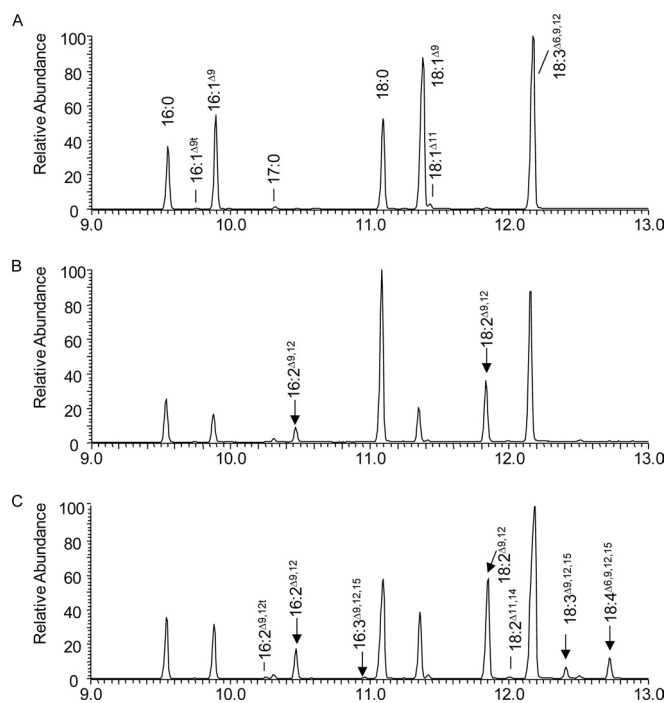

FIGURE 4. **Partial gas chromatogram of yeast feeding for CeFAT-2 with C18:3$^{\Delta 6,9,12}$.** *A–C*, FAMEs of yeast S288C expressing pYES2 vector only, AtFAD2, and CeFAT-2, respectively, fed with C18:3$^{\Delta 6,9,12}$.

$\Delta 6,9,12,15$ by DMOX adducts, by the gap of 12 atomic mass units with characteristic molecular ion at *m/z* 166, 206, 246 and 286, respectively.

Other yeast feeding experiments also suggest that $C18:1^{\Delta6}$, $C18:1^{\Delta9trans}$, $C18:1^{\Delta11}$, $C18:1^{\Delta12}$, $C18:1^{\Delta13}$, $C18:3^{\Delta9,12,15}$, $C20:1^{\Delta11}$, and $C20:2^{\Delta11,14}$, $C20:3^{\Delta8,11,14}$, $C20:4^{\Delta8,11,14,17}$ are not accepted as substrates by CeFAT-2 for further desaturation (data not shown). This suggests that the preexistence of a cis-$\Delta9$ double bond is required for second or third double bond formation, and $C18:3^{\Delta9,12,15}$ is not used for further desaturation at other positions (see supplemental Fig. S5).

A feeding experiment with $C16:1^{\Delta7}$ results in a low amount (1.3%) of $C16:2^{\Delta7,10}$ at a conversion rate of 3%, close to 12 times lower than converting $C16:1^{\Delta9}$ to $C16:2^{\Delta9,12}$. However, this low activity is not specific for CeFAT-2 because AtFAD2 also produces a trace amount (0.3%) of $C16:2^{\Delta7,10}$ at a conversion rate of 0.7% (supplemental Table S1). The identity of $C16:1^{\Delta7}$ and $C16:2^{\Delta7,10}$ peaks is confirmed by MS analysis on both FAMEs and DMOX adducts of the FAMEs which display mass spectra similar to the standard $C16:1^{\Delta7}$ and $C16:2^{\Delta7,10}$ (data not shown).

$C20:5^{\Delta5,8,11,14,17}$ ($\omega3$) rather than $C18:3^{\Delta9,12,15}$ is a major polyunsaturated fatty acid in *C. elegans*, thus it is of interest to know whether CeFAT-2 contributes to the $C20:5^{\Delta5,8,11,14,17}$ production. CeFAT-1 is the $\omega3$-desaturase converting $\omega6$ fatty acid $C20:4^{\Delta5,8,11,14}$ to $\omega3$ fatty acid $C20:5^{\Delta5,8,11,14,17}$. We show in yeast cells that CeFAT-1 is able to produce $C20:5^{\Delta5,8,11,14,17}$ from fed $C20:4^{\Delta5,8,11,14}$ at a conversion rate of 18%, but only a trace amount (0.44%) of $C20:5^{\Delta5,8,11,14,17}$ can be produced by CeFAT-2 at conversion rate of 2% (supplemental Table S2). This low desaturation activity is more a $\omega3$ desaturation, rather than a $\Delta15$ desaturation.

In addition, we have fed the yeast cells expressing these genes with $\omega3$ fatty acid $C20:4^{\Delta8,11,14,17}$ to check whether CeFAT-2 can produce $\omega3$ fatty acid $C20:5^{\Delta5,8,11,14,17}$ from this substrate. This $\Delta5$ desaturation step is catalyzed by FAT-4 in *C. elegans* (4). As expected, neither CeFAT-2 nor CeFAT-1 can use this substrate to produce $C20:5^{\Delta5,8,11,14,17}$.

Relationship of CeFAT-2 to $\Delta12$- and $\Delta15$-Desaturases—As expected, a BLAST search with the CeFAT-2 sequence identifies a diverse range of desturases, including $\Delta12$- and $\Delta15$-desaturases, in the NCBI data base. Alignment of the desaturase sequences shows that the nematode CeFAT-2 and CeFAT-1 form a separate cluster with bacteria or chloroplast $\Delta12$-desaturases (FAD6), whereas microsomal $\Delta12$-desaturases (FAD2) and $\Delta15$-desaturases (FAD3) form the other two major clusters (Fig. 5). However, CeFAT-2 shares a slightly higher sequence identity to AtFAD3 (29%) than AtFAD2 (27%).

We have also examined whether *C. elegans* $\Delta15$-desaturase CeFAT-1, when expressed in yeast under the same experimental conditions as CeFAT-2, has any ability to carry out a $\Delta12$ desaturation. CeFAT-1 was confirmed as $\omega3$-desaturase when expressed in yeast cells (6) and in plant (5) and is able to act on C16–C20 substrates. When we express CeFAT-1 under *Gal1* promoter in yeast S288C without the addition of exogenous substrate, no $\Delta12$-desaturase activity on $C18:1^{\Delta9}$ is detected (see supplemental Table S3). Additionally, when fed with $C18:2^{\Delta9,12}$, $C18:3^{\Delta6,9,12}$, or $C20:4^{\Delta5,8,11,14}$ substrates, CeFAT-1 clearly shows $\omega3$-desaturase activity resulting in the production of $C18:3^{\Delta9,12,15}$, $C18:4^{\Delta6,9,12,15}$, or $C20:5^{\Delta5,8,11,14,17}$. In addition, CeFAT-1 showed no strong preference for C20 over C18 fatty acid substrate as speculated elsewhere (1) because their

observed $\omega3$ desaturation rates were 7.1% and 9.5%, respectively (see supplemental Table S3).

DISCUSSION

The *C. elegans* CeFAT-2 was isolated and characterized as a nematode fatty acid $\Delta12$-desaturase in an earlier study (3). In the study, the $\Delta12$-desaturase activity of CeFAT-2 was confirmed by heterologous expression with the constitutive *ADH1* promoter in *S. cerevisiae* strain YRP685 (*MATa, leu2, lys2, his4, trp1, ura3*), where CeFAT-2 was able to desaturate $C16:1^{\Delta9}$ and $C18:1^{\Delta9}$ to produce $C16:2^{\Delta9,12}$ and $C18:2^{\Delta9,12}$. The study concluded that CeFAT-2 was a $\Delta12$-desaturase, and no obvious $\Delta15$ desaturation activity was reported (3). In contrast, we find that when expressed in yeast strain S288C the *C. elegans* $\Delta12$-desaturase CeFAT-2 can function as a $\Delta12/\Delta15$ bifunctional desaturase and behaves as a $\nu +3'$ desaturase (*i.e.* able to insert double bonds three carbons away from an existing double bond). More specifically, our results show that this enzyme is able to further desaturate a diverse range of fatty acid substrates containing a preexisting $\Delta9$ double bond sequentially at the $\Delta12$ and $\Delta15$ positions. We were unable to find the product $C18:2^{\Delta9,15}$, indicating that the enzyme requires a preexisting double bond at the $\Delta12$ position to carry out desaturation at the $\Delta15$ position. A possible reason for the observed discrepancy between the earlier study and ours could be the different yeast promoters used to drive the expression of the CeFAT-2. For example, the earlier study (3) used a constitutive *ADH1* promoter, whereas we have used an inducible promoter, and the failure to detect bifunctional activity in the earlier study might be due to different level of CeFAT-2 protein made. In the earlier study, the accumulation level of $C18:2^{\Delta9,12}$ product was relatively lower, and this might have led to the further desaturated products not being detected. In addition, we also confirm that CeFAT-1, as reported previously (6), is a $\omega3$-desaturase which converts $\omega6$ fatty acids $C18:2^{\Delta9,12}$, $C18:3^{\Delta6,9,12}$, and $C20:4^{\Delta5,8,11,14}$ into $\omega3$ products $C18:3^{\Delta9,12,15}$, $C18:4^{\Delta6,9,12,15}$, and $C20:5^{\Delta5,8,11,14,17}$ and displays no $\Delta12$ desaturation activity. This result indicates that the bifunctional activity of CeFAT-2 observed in our expression system is unlikely to be an artifact of our experimental conditions.

When expressed in yeast, CeFAT-2 can use a range of substrates for desaturation. These include $C14:1^{\Delta9}$, $C15:1^{\Delta9}$, $C16:1^{\Delta9}$, $C16:2^{\Delta9,12}$, $C17:1^{\Delta9}$, $C18:1^{\Delta9}$, $C18:2^{\Delta9,12}$, and $C18:3^{\Delta6,9,12}$ which are converted into $C14:2^{\Delta9,12}$, $C15:2^{\Delta9,12}$, $C16:2^{\Delta9,12}$, $C16:3^{\Delta9,12,15}$, $C17:2^{\Delta9,12}$, $C18:2^{\Delta9,12}$, $C18:3^{\Delta9,12,15}$, and $C18:4^{\Delta6,9,12,15}$ at the $\omega2$, $\omega3$, $\omega4$, $\omega1$, $\omega5$, $\omega6$, $\omega3$, and $\omega3$ positions, respectively. The production of $\omega1$-C16:3, a highly unusual fatty acid containing a terminal double bond, is noteworthy. Production of $\omega1$-C16:3 fatty acid has recently been reported in the yeast by the ectopic expression of an amoeba bifunctional $\Delta12/\Delta15$-desaturase (9) or a fungal $\Delta12,\omega3$-desaturase (10) and sorghum root hair fatty acid desaturase SbDES3 (15). The CeFAT-2 desaturase hence adds to the growing list of desaturases displaying remarkable inherent elasticity in being able to catalyze the synthesis of fatty acids desaturated the at $\omega1$, $\omega3$, or $\omega6$ position.

The newly discovered $\Delta12/\Delta15$ bifunctional desaturation activity of CeFAT-2 partially overlaps with the $\Delta15$ desaturation activity of CeFAT-1. We show in yeast cells that CeFAT-1 is able to desaturate $\omega6$ fatty acids $C18:1^{\Delta12}$, $C18:2^{\Delta9,12}$, $C18:3^{\Delta6,9,12}$, and $C20:4^{\Delta5,8,11,14}$ to $\omega3$ fatty acids $C18:2^{\Delta12,15}$, C18:
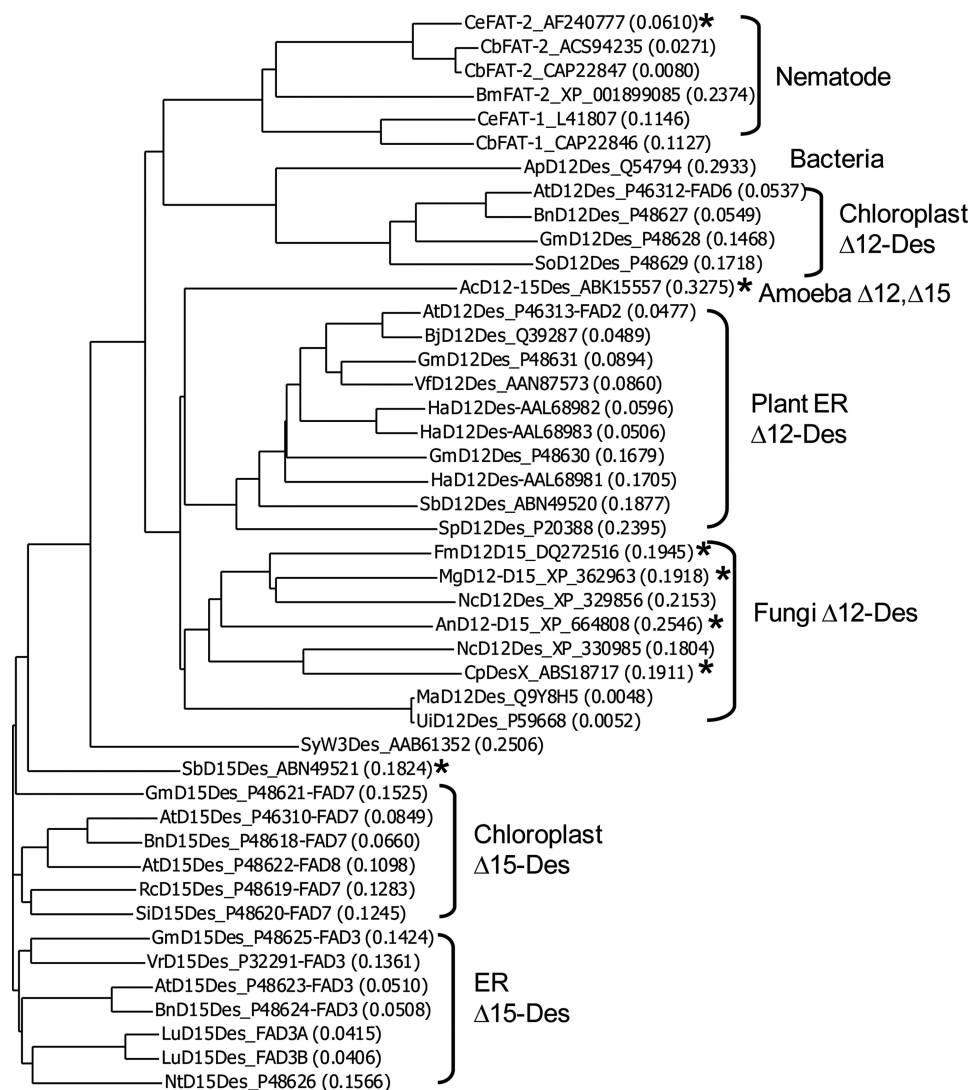
FIGURE 5. **Sequence comparison of CeFAT-2 and related desaturases.** Each sequence is followed by an accession number. * indicates bifunctional desaturase activity.

$3^{\Delta 9,12,15}$, C18:$4^{\Delta 6,9,12,15}$, and C20:$5^{\Delta 5,8,11,14,17}$, but only a trace amount (0.44%) of C20:$5^{\Delta 5,8,11,14,17}$ can be produced by CeFAT-2 from C20:$4^{\Delta 5,8,11,14}$ (supplemental Tables S2 and S3). From this additional feeding, we can conclude that the CeFAT-2 dual function does not substantially overlap with CeFAT-1, and it has only a minor ω3 desaturation activity. Therefore, it is unlikely that CeFAT-2 makes a substantial contribution to the synthesis of ω3 C20:$5^{\Delta 5,8,11,14,17}$ in *C. elegans*. The fact that the *C. elegan* *fat-1* mutant does not accumulate ω3 polyunsaturated fatty acids prompts the question whether CeFAT-2 is able to carry out any Δ15 desaturation in the nematode (4). Another possibility is that the expression level of CeFAT-2 is lower than CeFAT-1 in nematode, and as it only possesses up to 10% Δ15 desaturation activity compared with Δ12 desaturation activity, the CeFAT-2 Δ15 desaturation activity is not enough for complementing the *fat-1* mutation. The bifunctional Δ12/Δ15 characteristic of CeFAT-2 suggests the possible existence of an ancestral progenitor from which both CeFAT-2 and CeFAT-1 might have derived individually. It is thus possibly that CeFAT-2 evolved into a Δ12-desaturase but still maintained some

Δ15 desaturation activity, whereas CeFAT-1 evolved into a pure Δ15-desaturase. Alternatively, the nematode ancestor might have evolved the Δ12-desaturase from its Δ15-desaturase after a gene duplication event because the nematode *fat-1* and *fat-2* genes are transcribed in the same orientation, have similar structures of three exons and two introns, and are separated by ~5.3 kb of DNA (3). Finally, bifunctional Δ12/Δ15-desaturases have been isolated from fungi (7) and protozoa (9). Interestingly, these bifunctional desaturases (indicated by * in Fig. 5) cluster together with plant microsomal Δ12-desaturases and are separated from CeFAT-2, suggesting that CeFAT-2 has evolved independently from fungi or protozoa dual functional Δ12/Δ15-desaturases.

It will be of interest to identify the catalytic residues responsible for the Δ15 desaturation activity of Δ12-desaturase CeFAT-2. Meesapyodsuk *et al.* (10) identified two critical residues close to the conserved His boxes of *C. purpurea* Δ12/Δ15 bifunctional desaturase, Val-152 and Ala-206, which are conserved in the bifunctional desaturases but different from that of Δ12-desaturase. These authors confirmed the role of these residues as well as C-terminal amino acid sequence (residues 302–477) in regioselectivity

of the enzyme by site-directed mutagenesis or domain swapping. However, sequence alignment analysis suggests that CeFAT-2 is quite divergent from plant, fungi, or protozoa bifunctional $\Delta12/\Delta15$-desaturases, and the residues at the equivalent positions for the two key residues from *C. purpurea* $\Delta12/\Delta15$ bifunctional desaturase are different from both $\Delta12$-desaturase or $\Delta12/\Delta15$ bifunctional desaturases. Therefore, other residues may also be responsible for the newly confirmed $\Delta15$ desaturation activity of CeFAT-2. For example, residues close to His boxes conserved in either $\Delta15$-desaturases or $\Delta12/\Delta15$ bifunctional desaturases can be considered to be such candidates (supplemental Fig. 6S). Their role, if any, in the CeFAT-2 bifunctional activity remains to be studied further.

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