Construction and functional analysis of fatty acid desaturase gene disruptants in *Candida albicans*

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Polynsaturated fatty acids (PUFAs), including linoleic acid (C18:2) and α-linolenic acid (C18:3), are major components of membranes. PUFAs are produced from monounsaturated fatty acids by several fatty acid desaturases (FADs) in many fungi, but *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and humans do not have these enzymes. Although the fungal pathogen *Candida albicans* produces C18:2 and C18:3, the enzymes that synthesize them have not yet been investigated. In this report, two ORFs, *CaFAD2* and *CaFAD3*, were identified based on their homology to other yeast FADs, and *CaFAD2* and *CaFAD3* gene disruptants were constructed. *Cafad2Δ* and *Cafad3Δ* lost their ability to produce C18:2 and C18:3, respectively. Furthermore, *S. cerevisiae* cells expressing *CaFad2p* converted palmitoleic acid (C16:1) and C18:1 to hexadecadienoic acid (C16:2) and C18:2, respectively, and *CaFad3p*-expressing cells converted C18:2 to C18:3. These results strongly supported that *CaFAD2* encodes the Δ12 FAD and that *CaFAD3* encodes the ω3 FAD. However, phenotypic analysis demonstrated that the presence of these PUFAs did not affect the virulence to mice, or morphogenesis in the culture media used to induce morphological change of *C. albicans*.

**INTRODUCTION**

Fatty acids, whose principal function is to modulate the physical state of cell membranes, especially fluidity, are essential components for all organisms (Kitajima & Nozawa, 1996). Various environmental factors, such as temperature and nutrition, lead organisms to modify their fatty acid composition and maintain their optimal membrane fluidity in order to adapt to new environments. Cells grown at a lower temperature contain a larger proportion of polyunsaturated fatty acids (PUFAs) in their total lipids, and the increase in fatty acid unsaturation is thought to compensate for the decreased membrane fluidity resulting from the lower environmental temperature (Prasad et al., 1996).

PUFAs, including linoleic acid (C18:2) and α-linolenic acid (C18:3), are synthesized by a series of membrane-bound fatty acid desaturases (FADs) in eukaryotic cells. To begin with, the Δ9 FAD introduces a first double bond at the Δ9 position of both palmitic acid (C16:0) and stearic acid (C18:0), and converts them to monounsaturated fatty acids – palmitoleic acid (C16:1) and oleic acid (C18:1). The Δ12 FAD introduces a second double bond at the Δ12 position of C16:1 and C18:1, and converts them to hexadecadienoic acid (C16:2) and C18:2. Additionally, the ω3 FAD introduces a third double bond at the Δ15 position of C18:2 and converts it to C18:3 (Oura & Kajiwara, 2004). Generally, deletion of Δ9 FAD is lethal due to the organism’s inability to produce unsaturated fatty acids (UFAs).

In higher organisms, UFAs are known to play important roles as precursors of signalling molecules as well as structural molecules. PUFAs and their derivatives have been shown to act as developmental signals that induce asexual sporulation in some filamentous fungi (Calvo et al., 1999, 2001). Specifically, the Δ12 FAD mutant of the oilseed pathogen *Aspergillus parasiticus* has been reported to delay spore germination and to reduce conidia production (Wilson et al., 2004). However, Krishnamurthy et al. (2004) reported that a strain conditionally expressing *OLE1*, which encodes Δ9 FAD in the major fungal pathogen...
Candida albicans, was incapable of producing UFAs, and was defective in hyphal development and chlamydospore formation under repressed conditions. This indicated the involvement of PUFAs in morphogenesis.

C. albicans, which is responsible for candidiasis, causes life-threatening infection when host defences are impaired (Odds, 1987, 1988). The organism is a dimorphic yeast; it can exist as both yeast and filamentous mycelia. Both forms are pathogenic, but the mycelial forms penetrate tissues and because the molecular mechanisms of the dimorphic transition have not yet been understood clearly. Interestingly, the level of UFAs tended to be higher in the tissues and because both morphological forms are found in infected tissues and because the molecular mechanisms of the dimorphic transition have not yet been understood clearly. Interestingly, the level of UFAs tended to be higher in the mycelial form than in the yeast form (Sadamori, 1987; Yano et al., 1982; Ghannoun et al., 1986). There is immense interest in studying lipid pathways for several reasons: there are a limited number of effective and safe systemic antifungal drugs, lipids play an important role in pathogenicity and morphogenesis, and lipids provide potential targets for a novel class of antifungals.

Despite their importance, the enzymes that catalyse the synthesis of PUFAs are not well understood compared to Δ9 FAD. This is partially because the model yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe do not possess these enzymes that are involved in the reactions following the one by Δ9 FAD (Mishra et al., 1992). In addition, only three enzymes that desaturate the Δ9, Δ6 and Δ5 positions are detected in humans, in whom C18:2 and C18:3 are essential fatty acids (Nakamura & Nara, 2004).

In this study, we identified two genes in C. albicans that encode Δ12 and ω3 FADS, and named them CaFAD2 and CaFAD3, respectively. Moreover, in an effort to elucidate the functions of these enzymes, we constructed Cafad2Δ and Cafad3Δ null mutants. However, our results showed that an alteration in the composition of fatty acids resulting from the deletion of CaFAD2 or CaFAD3 did not affect morphogenesis or virulence under the conditions studied.

Table 1. Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>STA ste-VC9 ura3-52 trp1-289 his3-Δ1 leu2-3,112</td>
<td>IFO*</td>
</tr>
<tr>
<td>IFO10150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200</td>
<td>Kaneko et al. (2004)</td>
</tr>
<tr>
<td>TUA4</td>
<td>ura3Δ::imm434/ura3Δ::imm434 arg4::hisG200/ARG4 RP10::p3HA-Act1</td>
<td>Hanaoka et al. (2005)</td>
</tr>
<tr>
<td>TUA6</td>
<td>ura3Δ::imm434/ura3Δ::imm434 arg4::hisG200/ARG4::hisG200 Cafad2Δ::lhp200-URA3-hph200/Cafad2Δ::ARG4</td>
<td>This study</td>
</tr>
<tr>
<td>FAD2-2</td>
<td>Cafad2Δ::lhp200-URA3-hph200/Cafad2Δ::ARG4</td>
<td>This study</td>
</tr>
<tr>
<td>FAD3-2</td>
<td>ura3Δ::imm434/ura3Δ::imm434 arg4::hisG200/ARG4::hisG200 Cafad3Δ::lhp200-URA3-hph200/Cafad3Δ::ARG4</td>
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Fatty acid desaturases in Candida albicans

(5’-CCCTGTTGAAATTTGCTATCGTATGGATTGATTTACCTC-TTTATG-3’) and disFAD2-4 (5’-TTCACTTGACATAGATAGAATACAC-3’), respectively. Each fragment was used as a flanking homology region for a gene disruption cassette. For CaFAD3, two fragments, disFAD3-A and disFAD3-B, were amplified using primers disFAD3-1 (5’-ATCAATAACATAGAGATTTGATC-3’) and disFAD3-2 (5’-GTCGTAGCTGGGAAAACCCCTTGGCTGAAGATATTCAATACAGAAATG-3’), and disFAD3-3 (5’-CCCTGTTGAAATTTGCTATCGTATGGATTGATTTACCAGAGTCAATATG-3’), respectively. The PCR-amplified disruption cassettes containing an hph200 marker were transformed into the TUA4 arg4 ura3 strain. Finally, both alleles of the CaFAD2 and CaFAD3 loci were replaced with hph200 or ARG4, yielding the respective strains FAD2-2 and FAD3-2.

**Heterologous expression in S. cerevisiae.** The fragment containing CaFAD2 was isolated from pBS-CaFAD2, and ligated into the BamHl and SplI sites of the pYES2 expression vector Invitrogen). The resulting pYES2-CaFAD2 was transformed into S. cerevisiae IFO10150 by the lithium acetate method (Burke et al., 2000). The transformants were cultivated in SD-ura-rf medium overnight at 30°C. This overnight culture was transferred to fresh SD-ura-rf medium, and expression was induced by the addition of 1/100 volume 20% (w/v) galactose solution. The same strategy was used with SD-ura-rf medium supplemented with 1 mM C18:2 for CaFAD3 expression.

**Fatty acid analysis.** Total lipids were extracted from exponentially growing cells (OD600 1-0-1-5) with chloroform/methanol (1:2, v/v) and then methylated using 1% (v/v) sulfuric acid in methanol, as described previously (Watanabe et al., 2004). The resulting fatty acid methyl esters (FAMEs) were extracted in hexane and analysed by GLC (GC-18A, Shimadzu) with a 0.25 mm x 25 mm HR-SS-10 capillary column (Shinwa Chemical Industries). A FAME mixture used as a fatty acid standard was purchased from Sigma. Fatty acid 4, 4-dimethyloxazoline (DMOX) derivatives were prepared as described previously (Oura & Kajiwara, 2004). The derivatives were analysed by a GC mass spectrometer (QP5000; Shimadzu) operating at an ionization voltage of 70 eV, with a scan range of 40–400 kDa. The mass spectrum of any new peak obtained was interpreted by at an ionization voltage of 70 eV, with a scan range of 40–400 kDa.

**Animal study.** For each group, five male CD-1 (ICR) mice aged 4 weeks (Charles River Japan) weighing approximately 21–25 g were inoculated with 10⁶ c.f.u. by intravenous injection. Survival curves were determined by the Kaplan-Meier method and then were compared using the log-rank test. A P value of less than 0.05 was considered significant.

**RESULTS**

**Cloning and sequencing of the CaFAD2 and CaFAD3 genes**

Based on the ORFs released in the Candida genome database website (http://www.candidagenome.org/), we detected two sequences having high homology with Saccharomyces kluveri FAD2 and FAD3 proteins (SKFAD2p and SKFAD3p), respectively. The resulting two amino acid sequences, CaFAD2p (orf19-118) and CaFAD3p (orf19-4933), were predicted to be 437 and 434 aa polypeptides, respectively. The deduced amino acid sequence of CaFAD2p had the highest identity with that of SKFAD2p (63-1%), and the amino acid sequences of CaFAD3p and SKFAD3p had 63-2% identity (Fig. 1). With respect to the three histidine cluster motifs that are conserved among the D12 and ω3 FADs, and that are essential for FAD activity as potential ligands for iron atoms (Los & Murata, 1998), the motifs in both CaFAD2p and CaFAD3p are also highly conserved (Fig. 1).

**Gene disruption of CaFAD2 and CaFAD3**

To investigate the functions of the proteins encoded by the CaFAD2 and CaFAD3 genes, we constructed FAD2-2 (Cafad2A mutant) and FAD3-2 (Cafad3A mutant) strains of C. albicans, as described in Methods. Southern blotting and PCR amplification was used to confirm that the FAD2-2 and FAD3-2 mutants obtained were disrupted at the FAD2 and FAD3 loci, respectively, and that the growth rate of each mutant at 30°C in YPD medium was identical to that of the wild-type TUA6 strain (data not shown). The major fatty acids extracted from TUA6, FAD2-2 and FAD3-2 were determined by using GC analysis (Fig. 2a, b, c). No PUFA was detected in strain FAD2-2 (Fig. 2b), and C18:3 was not detected in strain FAD3-2 (Fig. 2c). The loss of PUFAs in each disruptant was restored by introducing a CaFAD2- or CaFAD3-expressing plasmid (data not shown). These results suggest that the CaFAD2 gene was involved in C16:2 and C18:2 syntheses, and that the CaFAD3 gene was involved in C18:3 synthesis.

**Heterologous expression of the CaFAD2 or CaFAD3 gene**

To examine whether or not CaFad2p functions as a Δ12 FAD, the CaFAD2 gene was expressed in S. cerevisiae IFO10150. Fatty acids were extracted from the S. cerevisiae strain harbouring pYES2-CaFAD2 and analysed by GLC. The resultant data demonstrated two unique major peaks in the CaFAD2-expressing strain (Fig. 3b), which were absent in the strain harbouring only the vector (Fig. 3a). These peaks were suggested to be derived from C16:2 and C18:2, according to the retention times of C16:2 and C18:2 methyl esters (9-3 and 13-3 min), respectively. Moreover, to determine the position at which the second double bond is induced, the GC-MS analysis of the fatty acid DMOX derivative suggested as C18:2 was performed. The mass spectrum of the fatty acid DMOX derivative exhibited a peak at m/z = 333, and the peaks produced by ionization at m/z = 196, 208, 236 and 248 were consistent with the double bonds at the Δ9 and Δ12 positions of the fatty acid (Fig. 4a). These results demonstrate that the CaFAD2 gene encodes the Δ12 FAD in C. albicans, and that the CaFad2p enables the S. cerevisiae cells to produce C16:2 and C18:2.

Next, we examined whether or not CaFad3p functions as an ω3 FAD in a similar way. The plasmid for CaFAD3 gene expression was introduced into S. cerevisiae IFO10150 and the expression of this gene was then induced in the presence of C18:2 as a substrate. The GC analysis of fatty acids...
Fig. 1. Comparison of the deduced amino acid sequences of Fad2p and Fad3p between *C. albicans* and *S. kluyveri*. Underlining indicates the histidine cluster motif. Sequences were aligned using the FASTA algorithm. CaFAD2 is used as the leading strand. Dots indicate amino acids identical to this reference sequence and alignment gaps are denoted by hyphens.

(a) (b) (c)

Fig. 2. GC analysis of fatty acids in total lipids extracted from *C. albicans* strains. The wild-type strain TUA6 (a), the Cafad2Δ disruptant (b) and the Cafad3Δ disruptant (c) were cultured in SD-arg-ura medium at 30 °C. C16:0, palmitic acid; C16:1, palmitoleic acid; C16:2, hexadecadienoic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, α-linolenic acid.
extracted from the *S. cerevisiae* strain harbouring pYES2-CaFAD3 exhibited an additional unique peak (Fig. 3d), which was absent when pYES2 was introduced in *S. cerevisiae* (Fig. 3c). This peak was derived from C18:3, according to the retention time (15 ± 1 min) of its methyl ester (Fig. 3d), the mass spectrum (*m/z* = 331) of its DMOX derivative, and the cleavage peaks at *m/z* = 196, 208, 236, 248, 276 and 288 (Fig. 4b), which were consistent with the double bonds at the D9, D12 and D15 positions of the fatty acid. These results revealed that the CaFAD3 gene encodes an ω3 FAD in *C. albicans* and that the CaFAD3 protein confers ability to produce C18:3 on *S. cerevisiae*.

**Phenotypic analysis of Cafad2Δ and Cafad3Δ disruptants**

To examine the cold-sensitivity of the mutants we examined the growth rate of both Cafad2Δ and Cafad3Δ, and the wild-type strain TUA6, at 10, 15 and 20 ºC in YPD media. The experiment was done twice. There was no significant difference in the OD₆₅₀ and c.f.u. among strains. To investigate the effects of PUFAs on morphogenesis in *C. albicans*, we observed the hyphal formation of both Cafad2Δ and Cafad3Δ mutants on solid media or in liquid media, such as Lee’s medium, Spider medium and serum medium, at 25, 30 and 37 ºC. The disruptant strains grew equally as well as the wild-type strain TUA6 under these conditions, and the hyphal formation of the disruptants was almost the same as that of TUA6 in all three media at all temperatures (data not shown). We also examined chlamydospore formation, an alternative morphogenetic event in *C. albicans*. For chlamydospore formation, cells were streaked on cornmeal agar with 1% (v/v) Tween 80 (Buckley *et al.*, 1982), covered with coverslips to maintain a semi-aerobic environment and incubated at 25 ºC for 7 days. The disruptant strains formed chlamydospores normally (data not shown), indicating that PUFAs do not contribute to the formation of hyphae or chlamydospores.

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**Fig. 3.** GC analysis of fatty acids in total lipids extracted from *S. cerevisiae* transformants. The *S. cerevisiae* IFO10150 strain harbouring the pYES2 vector (a) or pYES2-CaFAD2 (b) was cultured in SD-ura-raf medium, and the *S. cerevisiae* strain harbouring the pYES2 vector (c) or pYES2-CaFAD3 (d) was cultured in SD-ura-raf medium supplemented with 1 mM C18:2 at 30 ºC. A 1/100 volume 20% (w/v) galactose solution was added to each culture to induce protein expression. Arrowheads in (b) and (d) indicate the peaks specific to the CaFADs-expressing strains and was analysed by GC-MS (Fig. 4). C16:0, palmitic acid; C16:1, palmitoleic acid; C16:2, hexadecadienoic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, ω-3-linolenic acid.
To determine the role of PUFAs in virulence, mice were intravenously injected with \(10^6\) c.f.u. of the wild-type (TUA6), or the Cafad2\(^D\) (FAD2-2) or Cafad3\(^D\) null (FAD3-2) mutants, and monitored for survival. No significant difference in mortality was found (FAD2-2 vs TUA6, \(P = 0.73\); FAD3-2 vs TUA6, \(P = 0.31\)), suggesting that PUFAs were not associated with the systemic infection with \(C.\) albicans.

**DISCUSSION**

This is believed to be the first report on the cloning and characterization of \(\Delta 12\) and \(\omega 3\) FADs in the dimorphic fungus \(C.\) albicans. CaFad2p was found to share 63\% identity to SKFAD2p, the \(\Delta 12\) FAD of *Aspergillus nidulans* and the \(\Delta 12\) FAD of *A. parasiticus*, respectively; and CaFad3p was found to share 63\% similarity to SKFAD3p. However, CaFAD2p showed 59\% and 56\% identities with CaFad3p and SKFAD3p, respectively, percentages that are higher than those of the \(\Delta 12\) FAD of the other fungi. Therefore, it was difficult to decide whether each CaFAD gene is a \(\Delta 12\) FAD or an \(\omega 3\) FAD. The high homology among such FADs in some yeasts and other organisms has fascinated us, impelling our investigation of the enzymes of \(C.\) albicans. We have provided biochemical data indicating that CaFad2p or CaFad3p should function as only a \(\Delta 12\) or \(\omega 3\) FAD, respectively. However, we were unable to find any supporting evidence that PUFAs have some involvement in cold-sensitivity, morphogenesis in the media or virulence.

In some *Aspergillus* species, PUFAs and their derivatives act as developmental signals. For example, the mutants deleted of \(\Delta 12\) FAD show different physiological phenomena compared to the wild-type (Wilson *et al.*, 2004). Since similar functions of PUFAs are also expected in *C. albicans*, we disrupted the CaFAD2 and CaFAD3 genes. However, phenotypic characteristics such as germ tube formation, hyphal morphogenesis and chlamydospore formation (Nobile *et al.*, 2003) did not differ among the wild-type strain, Cafad2\(^D\) and Cafad3\(^D\) (data not shown), although it was reported that the *C. albicans* \(\Delta 9\) FAD gene (*OLE1*) affects viability and morphogenesis significantly (Krishnamurthy *et al.*, 2004). There may be small amounts of fatty acids in some media containing serum, which might complement the hypothesized defects involving PUFAs. But we were able to exclude this hypothesis by using a hyphae-inducing synthetic medium such as Spider medium. These facts suggested that C18 : 1, not PUFAs, plays a key role in morphogenesis or in signalling for the morphogenetic transition in *C. albicans*, unlike the case with *Aspergillus* species. In fact, some yeasts,

![Fig. 4. GC-MS analysis of the DMOX derivative. The spectra of the DMOX derivative of the fatty acids indicated in Fig. 3. (a) linoleic acid derived from *S. cerevisiae* strain harbouring pYES2-CaFAD2 and (b) \(\omega 3\)-linolenic acid derived from the strain harbouring pYES2-CaFAD3. The deduced structure of the fatty acid derivative is shown above the spectrum.](image-url)
like S. cerevisiae and Schizosaccharomyces pombe, do not have FADs other than Δ9 FAD, and do not require PUFAs for vegetative growth (Ratledge & Evans, 1991).

In infected tissues, the mycelial form of C. albicans predominates. The major differences in the lipid composition between the two morphological forms, yeast and hyphae, are alterations in phospholipids and fatty acid composition (Yano et al., 1982). The transition into the hyphal form causes a great increase in linoleic acid, contained in all phospholipids, the presence of which accompanies a compensatory decrease in monounsaturated fatty acids. These alterations point to a higher degree of unsaturation. Unexpectedly, in our study there were no obvious differences in virulence to mice between the wild-type strain and the Cafad2Δ or Cafad3Δ disruptants. However, the mice were fed bait including fatty acids, and C. albicans is able to assimilate fatty acids from the cells of mice. Thus, in the future, animal studies using the C. albicans mutant that is unable to assimilate exogenous fatty acids are necessary in order to clarify the relationship between PUFAs and the virulence of this yeast.

The expression levels of FAD3 and FAD2 in S. kluveri increased at low temperatures (Watanabe et al., 2004, Oura & Kajiwara, 2004). In C. albicans, the proportion of PUFAs at 25°C has been shown to exceed that at 37°C (Brondz & Olsen, 1990), suggesting the CafAD2 and CafAD3 enzymes might be involved in membrane fluidity or some other functions. Generally, higher temperatures increase membrane fluidity. The functional state of the lipid bilayer is a liquid-crystalline phase, but a decrease in temperature induces a gel-phase transition and a drastic loss of membrane fluidity. This phase transition essentially depends on the lipid composition of the membrane, especially the fatty acyl chains. The cis-unsaturated double bond in the chain induces a 30° bend, which creates a cavity in the lipid layer and perturbs the packing density (Feller & Gerday, 2003). Further studies under stressed growth conditions, such as at low temperatures or limited nutrition, e.g. in the human body, may unveil the hidden roles of FADs in C. albicans.

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REFERENCES


