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

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Polyunsaturated 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. *CaFAD2* Δ and *CaFAD3* Δ 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 $\Delta 12$ FAD and that *CaFAD3* encodes the $\omega 3$ 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*.

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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 $\Delta 9$ FAD introduces a first double bond at the $\Delta 9$

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 $\Delta 12$ FAD introduces a second double bond at the $\Delta 12$ position of C16:1 and C18:1, and converts them to hexadecadienoic acid (C16:2) and C18:2. Additionally, the $\omega 3$ FAD introduces a third double bond at the $\Delta 15$ position of C18:2 and converts it to C18:3 (Oura & Kajiwar, 2004). Generally, deletion of $\Delta 9$ 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 $\Delta 12$ 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 $\Delta 9$ FAD in the major fungal pathogen

Abbreviations: DMOX, 4,4-dimethyloxazoline; FAD, fatty acid desaturase; PUFA, polyunsaturated fatty acid; UFA, unsaturated fatty acid.

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 more easily and are, therefore, considered to be more pathogenic (Sobel *et al.*, 1984). However, the pathogenic roles of morphological changes remain to be elucidated, 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; Ghannoum *et al.*, 1986). There is immense interest in studying lipid pathways for several reasons: there are a limited number of effective and safe systemic anti-fungal 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 $\Delta 9$ 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 $\Delta 9$ FAD (Mishra *et al.*, 1992). In addition, only three enzymes that desaturate the $\Delta 9$, $\Delta 6$ and $\Delta 5$ 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 $\Delta 12$ and $\omega 3$ FADs, and named them *CaFAD2* and *CaFAD3*, respectively. Moreover, in an effort to elucidate the functions of these enzymes, we constructed *Cafad2* Δ and *Cafad3* Δ 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.

METHODS

Strains, plasmids and growth conditions. The yeast strains used and generated in this work are listed in Table 1. We used *Escherichia coli* DH5 α and the cloning vector pBluescript II KS(–) (Stratagene) for DNA manipulation. The expression vector for *S. cerevisiae*, pYES2, was purchased from Invitrogen. The expression vector pFLAG-Act1 (Umeyama *et al.*, 2002) was used for gene expression in *C. albicans*. Yeast strains were grown routinely at 30 °C in YPD medium (1 % w/v yeast extract, 2 % w/v bacto-peptone, 2 % w/v glucose) or YNB medium. The YNB media consisted of the following: SD-ura [0.67 % w/v Difco yeast nitrogen base without amino acids, 2 % w/v glucose, 0.77 g l^{–1} complete supplement mixture (CSM)-ura (Q-Biogene)], SD-arg-ura [having the same composition as SD-ura except with 0.72 g l^{–1} CSM-arg-ura (Q-Biogene) instead of CSM-ura], SD-ura-raf (having the same composition as SD-ura supplemented with 2 % w/v raffinose). The *Candida* strains (Ura[–]) were grown in YPDU medium [YPD medium supplemented with uridine (100 µg ml^{–1})]. Induction of filamentation in *C. albicans* was performed on the following solid media: Lee’s medium (Lee *et al.*, 1975), Spider medium (Liu *et al.*, 1994) and serum medium (2 % w/v agar containing 10 % v/v calf serum).

Cloning and DNA sequencing of *C. albicans* FAD genes. The *CaFAD2* coding sequence was amplified by PCR using the primers FAD2-5’ (5’-GCCGGATCCAGGATGGCAGCTGCTACAACGTC-3’) and FAD2-3’ (5’-GGCGCATGCTCAATGGGTTTGCTTTTAGGATCGAC-3’) (*Bam*HI or *Sph*I site is underlined), and then ligated into the *Eco*RV site of the cloning vector pBluescript II KS(–) (Stratagene), generating pBS-*CaFAD2*. The plasmid pBS-*CaFAD3* was constructed in a similar way. The *CaFAD3* ORF was amplified with the primers FAD3-5’ (5’-GCCGGATCCAGGATGAGTGTAGT-TGAGGCATCATCAAG-3’) and FAD3-3’ (5’-GGCGCATGCTTAA-TCTTTTGGTTTAACTGGTCC-3’). Both strands were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3100-Avant genetic analyser (Applied Biosystems).

Gene disruption in *C. albicans*. Gene disruption was performed using a method similar to that described by Hanaoka *et al.* (2005). Briefly, for *CaFAD2*, two fragments, disFAD2-A and disFAD2-B, were amplified using primers disFAD2-1 (5’-GATAGATGACTTCTT-TGGATGAAC-3’) and disFAD2-2 (5’-GTCGTGACTGGGAAAAC-CCTGGCGAATAATTATTACAACCTGATTGAAT-3’), and disFAD2-3

Table 1. Yeast strains

Strain	Genotype	Source
<i>S. cerevisiae</i>		
IFO10150	MATa ste-VC9 ura3-52 trp1-289 his3-Δ1 leu2-3,112	IFO*
<i>C. albicans</i>		
TUA4	ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200	Kaneko <i>et al.</i> (2004)
TUA6	ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/ARG4 RP10::p3HA-Act1	Hanaoka <i>et al.</i> (2005)
FAD2-2	ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200	This study
	Cafad2Δ::hph200-URA3-hph200/Cafad2Δ::ARG4	
FAD3-2	ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200	This study
	Cafad3Δ::hph200-URA3-hph200/Cafad3Δ::ARG4	

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(5'-CCTGTGTGAAATTGTTATCCGCTCTATTGATTGATTTACTTC-TTTATG-3') and disFAD2-4 (5'-TTCACCTGAACATATAGATAATAC-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'-ATCAATAACAATAGAGATTTGTAC-3') and disFAD3-2 (5'-GTCGTGACTGGGAAAACCCCTGGCGTGCAAGATTCAATAACAAGAAATG-3'), and disFAD3-3 (5'-CCTGTGTGAAATTGTTATCCGCTCTATTGATTGATTTACTTCTTTATG-3') and disFAD3-4 (5'-TGTGGTTTTACCAGAGTCAATATG-3'), respectively. The PCR-amplified disruption cassettes containing an *hph200-URA3-hph200* or *ARG4* marker were transformed into the TUA4 *arg4 ura3* strain. Finally, both alleles of the *CaFAD2* and *CaFAD3* loci were replaced with *hph200* and *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 *Bam*HI and *Sph*I 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-raf medium overnight at 30 °C. This overnight culture was transferred to fresh SD-ura-raf 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-raf 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 were 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 × 25 m 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 & Kajiura, 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 comparing it to that of the DMOX derivative of C18:2 or C18:3, prepared from C18:2 or C18:3 methyl ester (Sigma), and to the published value (reviewed by Spitzer, 1997).

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 calculated using 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 kluyveri* 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 $\Delta 12$ and $\omega 3$ 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 (*Cafad2Δ* mutant) and FAD3-2 (*Cafad3Δ* 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 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 $\Delta 12$ 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 $\Delta 9$ and $\Delta 12$ positions of the fatty acid (Fig. 4a). These results demonstrate that the *CaFAD2* gene encodes the $\Delta 12$ 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 $\omega 3$ 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

CaFAD2	1	MAAATTSFSSGFNNNNADQSTDSSATISKSGNVASFKTTSTTSTYQTNLTAIDTYGNEFKVPDYTIKDILSAIP	75
SkFAD2	1	-----MSAVTVT.SDPKNRGS.SNTEQEVPKV...N..V.S...F.....G...	51
CaFAD3	1	-----MSVVE.SS.SVVEDSTASNVVQGRNISSFAST.ASS...T...N.KV.....S.....Q...	63
SkFAD3	1	-----MSIETVGS.SGVAIN.KAVS..AT.VVQPK....N..V.....	55
CaFAD2	76	THCYERRLLQSLSYVFRDIFCMVVLGFIANNYIHLI-----PN-QF-IRFAAWTGYVWCQGLFGTGIWVLAHECG	143
SkFAD2	52	HE.....AT..Y.....LTT.YLTHKILYPL-LISYTSNSI-.K.TF.AL.TYV.....	124
CaFAD3	64	K.....S.IR..G..V...TM..II.YVGHTF.PMVQIPEY.TLAYGL.G.L.MVQSY.I...F.L.I.....	138
SkFAD3	56	KE..K.DT.W..H..V...AAIL.I.YLGT...PVL----F..SAL-L.GI.YAIQSYLI...F.L.I.....	125
CaFAD2	144	HQAFSDYGSVNDVFGWVLHSYLLVPYFSWKFSHGKHHKATGHLTRDMVFPKTKEEFLQNRGV-KDLDLGDSP	217
SkFAD2	125	I.....T....M.....Y.....M.....A.....KKS.NFFGN.AEYSE...	199
CaFAD3	139	.G.....QNI...I..I.....I.....A.....K...I.Y...Y.EKNK.-EKVA..MEE..	212
SkFAD3	126	.S...ESNA...T.....WWM...P....S.....M.....I.Y..D..ITMKKK-SKFAEITEEA.	199
CaFAD2	218	MYSLLTLIFQQTFGWISYLVANVSGQKYPGVSFKLNFNPNSLIFDKKDYWYILLSDLGILLQFFNLYVWYQSF	292
SkFAD2	200	LRT.YE.LV..LG...A..FV..T..P..D.PSW.W...WLT.PL.EQR.AL..F.....T.GIV.TL..KK.	274
CaFAD3	213	I..F.V.V...LG.LQL..AT.AT..V...Y.KIAKS.YT.T.PV...HQ...V...I..A.TTV.Q..KN.	287
SkFAD3	200	VMT.FN..A..VG.LQL..AT.AT..P....KKFFKS.YW.T.PV..A..F.W.IM..I..VSTLLIN.L..RAY	274
CaFAD2	293	GGFNLLVNYVLPYFLVNHVLVFITYLQHSDPQMPHYEASQWTFARGAAATIDREFGVGKHIFHDIETHVLHHY	367
SkFAD2	275	..WS.FI.WFV..IW.....F...T..T...N.EE...K.....K...I.P.....	349
CaFAD3	288	.L..MMI.WFV.WLW.....V.F...T..T...TSKE.....N....Q.....	362
SkFAD3	275	.AHVV.I.WFI.WLW.....V.F...T..T...D.EE...K.....N....Q.....	349
CaFAD2	368	VSRIPIFYNAREASEAIKKVMGIHYQHSDENMWVSLWKSARWCQFVDGN---NGVLMYRNTNGFGVDPKKQTH--	436
SkFAD2	350	C.....P.....K..RS.....K....F.S..Y..D-----F..I.NC..GAAEK----	416
CaFAD3	363	TD..R....E..RYEG.S..Y...CM.M.....DDKEDAK..M.F..V..W.PVKP.D----	433
SkFAD3	350	C.....K.TS...E...Q..RYEG....K...V..S..Y.E.D-----R.F....V.-VKPEDGSSQ	419

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.

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.

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 °C. 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, 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 $\Delta 9$, $\Delta 12$ and $\Delta 15$ positions of the fatty acid. These results revealed that the *CaFAD3* gene encodes an $\omega 3$ FAD in *C. albicans* and that the *CaFAD3* protein confers ability to produce C18:3 on *S. cerevisiae*.

Phenotypic analysis of *Cafad2* Δ and *Cafad3* Δ disruptants

To examine the cold-sensitivity of the mutants we examined the growth rate of both *Cafad2* Δ and *Cafad3* Δ , and the wild-type strain TUA6, at 10, 15 and 20 °C in YPD media.

The experiment was done twice. There was no significant difference in the OD₆₁₀ and c.f.u. among strains. To investigate the effects of PUFAs on morphogenesis in *C. albicans*, we observed the hyphal formation of both *Cafad2* Δ and *Cafad3* Δ mutants on solid media or in liquid media, such as Lee's medium, Spider medium and serum medium, at 25, 30 and 37 °C. 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 °C 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.

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) α -linolenic acid derived from the strain harbouring pYES2-CaFAD3. The deduced structure of the fatty acid derivative is shown above the spectrum.

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* Δ (FAD2-2) or *Cafad3* Δ 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 63.1, 55.7 and 54.7 % 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 63.2 % similarity to *SkFad3p*. However, *CaFad2p* showed 59.3 and 56.3 % 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 chlamyospore formation (Nobile *et al.*, 2003) did not differ among the wild-type strain, *Cafad2* Δ and *Cafad3* Δ (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,

like *S. cerevisiae* and *Schizosaccharomyces pombe*, do not have FADs other than $\Delta 9$ 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 *Cafad2* Δ or *Cafad3* Δ 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. kluyveri* increased at low temperatures (Watanabe *et al.*, 2004, Oura & Kajiwar, 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 properties. 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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