Dosage-dependent functions of fatty acid desaturase Ole1p in growth and morphogenesis of Candida albicans

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Conditions in the infected human host trigger virulence attributes of the fungal pathogen Candida albicans. Specific inducers and elevated temperatures lead to hyphal development or regulate chlamydospore development. To explore if these processes are affected by membrane lipids, an investigation of the functions of the Ole1p fatty acid desaturase (stearoyl-CoA desaturase) in C. albicans, which synthesizes oleic acid, was undertaken. A conditional strain expressing OLE1 from the regulatable MET3 promoter was unable to grow in repressing conditions, indicating that OLE1 is an essential gene. In contrast, a mutant lacking both alleles of OLE2, encoding a Ole1p homologue, was viable and had no apparent phenotypes. Partial repression of MET3p–OLE1 slightly lowered oleic acid levels and decreased membrane fluidity; these conditions permitted growth in the yeast form, but prevented hyphal development in aerobic conditions and blocked the formation of chlamydospores. In contrast, in hypoxic conditions, which trigger an alternative morphogenetic pathway, hyphal morphogenesis was unaffected. Because aerobic morphogenetic signalling and oleic acid biosynthesis require oxygen, it is proposed that oleic acid may function as a sensor activating specific morphogenetic pathways in normoxic conditions.

INTRODUCTION

The fluidity of cellular membranes is determined to a large extent by their lipid composition and by ambient temperatures. High levels of unsaturated fatty acids, low amounts of sterols in eukaryotic membranes and high temperatures increase fluidity (Carratu et al., 1996; Chatterjee et al., 1997; Horvath et al., 1998). It has been proposed that changes in the physical state of membranes are directly sensed and transmitted by specific signalling pathways triggering protective stress responses (Moskvina et al., 1999). During a heat shock such membrane-induced events could contribute to the complete set of stress responses, which alternatively are activated by protein unfolding (Ananthan et al., 1986; Torok et al., 1997). In yeast an increase in levels of saturated fatty acids within membrane lipids lowered the response to heat shock (Carratu et al., 1996). Similarly, artificially increasing or lowering membrane fluidity in cyanobacteria lowered and, respectively, increased the set point of the heat-shock response (Horvath et al., 1998; Wada et al., 1990; Vigh et al., 1993). Several organisms adapt to low temperatures by increasing the degree of fatty acid desaturation, which leads to an increase in membrane fluidity and simultaneously increases responses to elevated temperatures (Cossins, 1994; Vigh et al., 1998).

In the yeast Saccharomyces cerevisiae the OLE1 gene product encodes a stearoyl-CoA desaturase (EC 1.14.99.5) located in membranes of the endoplasmic reticulum (ER), which transforms the CoA derivatives of palmitic and stearic acid into the corresponding Δ9 monounsaturated palmitoleic (C16:1) and oleic acid (C18:1) derivatives, respectively (Stukey et al., 1989). The C16:1 derivative is the predominant unsaturated fatty acid in S. cerevisiae, while other fungi including Candida species also produce C18:2 (linoleic) and C18:3 (linolenic) fatty acids, which are not present in S. cerevisiae (reviewed by Mishra et al., 1992). Fatty acid desaturases use electrons in cytochrome b5 (derived from NADH by NADH-dependent cytochrome b5 reductase) and molecular oxygen to form a double bond between C-atoms 9 and 10 of fatty acids. Remarkably, fungal fatty acid desaturases contain a cytochrome b5 domain as integral parts of their enzyme structures, while vertebrate enzymes use separate cytochrome b5 molecules (Mitchell & Martin, 1995). The active site in the native protein comprises three histidine-rich sequences, which fold to form two iron-binding sites. Yeast Ole1p contains four putative transmembrane regions, which by forming two pairs of membrane-traversing regions could attach the desaturase to the ER membrane and leave most of the
Olpe sequences within the cytoplasm (Stukey et al., 1990). OLE1 expression is regulated by fatty acids, oxygen and temperature. Saturated fatty acids induce a 1-6-fold increase in transcription, while unsaturated fatty acids repress OLE1 transcription up to 60-fold (McDonough et al., 1992; Bossie & Martin, 1989). At low temperatures and during oxygen limitation, OLE1 expression is induced (Kwast et al., 1998; Nakagawa et al., 2002). A deletion analysis of the OLE1 promoter identified a 111 bp fatty acid-regulated region (FAR) which is essential for transcription activation and repression by unsaturated fatty acids (Choi et al., 1996).

In addition, the low oxygen response promoter element (LORE) mediates oxygen repression of OLE1 (Nakagawa et al., 2001; Vasconcelles et al., 2001). Two genes encoding components of fatty acid transporters, FAA1 and FAA4, were found to be essential for unsaturated fatty acid repression of OLE1 via FAR sequences (Faergeman et al., 2001). Also, the acyl-CoA binding protein and the Snm1–Tup1 complex were shown to be involved in repression of OLE1 (Fujimori et al., 1997). On the other hand, the Hap1 transcriptional activator (Choi et al., 1996) and two transcription factors, Spt23 and Mga2p, which are initially naturally short-lived enzyme and is degraded by ubiquitin/proteasome-dependent ER-associated degradation (Braun et al., 2002). Lowering of Ole1p activity leads to a loss of mitochondrial inheritance (Stewart & Yaffe, 1991) and disturbs the integrity of the nuclear membrane (Zhang et al., 1999), although growth is not affected even if unsaturated fatty acid levels are reduced down to one-eighth of the normal level (Stukey et al., 1989).

The human fungal pathogen Candida albicans is able to assume different growth forms, which appear to have different roles for host–cell interaction and virulence (reviewed by Ernst, 2000). At body temperatures and in the presence of inducing agents, a true hyphal form is induced, which may be involved in anchoring within and the presence of inducing agents, a true hyphal form is effectively prevents the formation of hyphal filaments and chlamydospores in aerobic conditions. Our results indicate, however, that overall membrane fluidity is not directly responsible for the morphogenetic potential of C. albicans, but that oleic acid has a specific role to activate specific morphogenetic pathways.

### METHODS

#### Strains and growth conditions.

C. albicans strains used in this study are listed in Table 1. Transformed strains were generated as described by Wilson et al. (1999). Strains were routinely grown in YPD or SD medium (Sherman et al., 1986). A 1% solution of the non-ionic detergent Igepal CA-630 (Sigma) was used for solubilization of 0.5 mM oleic acid in media (Stukey et al., 1989). Strains were incubated in microaerophilic conditions by using a CampyGen bag in anaerobic jar (Oxoid). The PCK1 promoter (PCK1p) was induced in SCAA medium (0-67% yeast nitrogen base without amino acids and 2% Casamino acids) or SL medium (0-67% yeast nitrogen base, 2% sodium lactate) and repressed in SD medium (Leuker et al., 1997). To repress the MET3 promoter (MET3p), SD medium supplemented with different concentrations of methionine and/or cysteine was used (Care et al., 1999).

Strains were grown for 3–4 days at 37 °C on Lee’s medium (Lee et al., 1975), or on Spider-Plates (Liu et al., 1994) or on 5% horse serum solidified by 2% agar to induce hyphae. Corn meal agar (CMA) (Difco) containing 0-33% Tween 80 was used for chlamydospore induction (Joshi et al., 1993). Strains were streaked lightly on the agar surface and covered by coverslips. Following incubation at room temperature for 5 days, photographs of chlamydospores and filaments were taken with a Zeiss Axioskop microscope across the coverslips.

S. cerevisiae Y0779 (Ade1::LEU2 leu2-3,112 lys2-801 trpl-1 ura3-52), kindly provided by S. Jentsch, was cultured at 30 °C in SD medium supplemented with 0-2% oleic acid in 0-2% NP40 (Braun et al., 2002). It was transformed by expression plasmids containing OLE1 (pSKM24) or OLE1-GFP (pSKM62) under transcriptional control of the C. albicans PCK1 promoter.

#### Chromosomal deletions of OLE1 and OLE2.

C. albicans sequence data were obtained from the Stanford Genome Technology Center website (http://www-sequence.stanford.edu/group/candida). The genomic region of OLE1 was isolated by PCR using DNA of strain CAI4. The entire OLE1 coding region was disrupted by the Ura-blaster method (Fonzi & Irwin, 1993). A cassette for disruption of OLE1 was constructed in several steps. First, sequences 5′ and 3′ of the OLE1 ORF were amplified by PCR and subcloned. A fragment (0-98 kb) of the 5′ sequences flanking the start of the CaOLE1 ORF was amplified using primers Ole1dsA and Ole1dsB (5′-CTAGAAGCCTACGATGCGAGCT-3′/5′-CTAGAGCTGACGATCGATCC-3′; bold, regions of homology; italics, BamHI); similarly, 728 bp of the 3′ untranslated sequences were amplified using primers Ole1dsC and Ole1dsD (5′-CTAGAGCTGACGATGCGAGCT-3′/5′-CTAGAGCTGACGATC-3′; bold, regions of homology; italics, PstI). PCR fragments were subcloned into pUC18, which resulted in plasmids pSKM8 and pSKM7, respectively. The BamHI fragment of pSKM8 was cloned into the BglII site of pSK91. The PstI fragment of pSKM7 was inserted into the PstI site of the resulting plasmid pSKM55. A plasmid with OLE1 flanking sequences in the correct orientation was obtained (pSKM56). Its HindIII fragment containing the OLE1 disruption cassette was used to transform strain CAI4.

OLE2 was disrupted similarly by subcloning the regions flanking and partially including the ORF. The 876 bp 5′ region was amplified
by genomic PCR using primers 709 and 710 (5′-TATGGATCCCAAA-ACTCCCTGTAAGATGG-3′/5′-TATGGATCCCATACAAAGCTGC-3′; bold, regions of homology; italics, BamHI); the 870 bp 3′ region was amplified by genomic PCR using primers 711 and 712 (5′-TATAGATCTGAGGGTTG-3′/5′-TATAGATCTGGCAACTTTTCTTATTGC-3′; bold, regions of homology; italics, BglII). The BamHI fragment carrying the 5′ region was subcloned into the BglII site of pSKM24, while the BglII fragment carrying the 3′ region was subcloned into the BamHI site of the resulting vector, to construct pAP9A. The SacI–SphI fragment of pAP9A was used for sequential disruption of both OLE2 alleles (Fonzi & Irwin, 1993).

To place OLE1 under control of the MET3 promoter we followed a previously described strategy (Care et al., 1999). First, a fragment of 624 bp corresponding to the 5′ end of the OLE1 ORF was amplified by PCR using the primers OleBHI-Nterm (5′-CTTTAGCTGATGCCATG-3′) and OleSpBHI-HindIII (5′-CTTTAGCTGGATCCACTTTGG-3′); bold, regions of homology; underlining, HindIII, BamHI) and the PCR fragment was cloned into pUC18, resulting in plasmid pSKM20. The BamHI fragment of pSKM20 was inserted into the single BamHI site of pCaDis, downstream of the MET3 promoter (Care et al., 1999). The resulting plasmid pSKM25 was linearized by NcoI (which cuts within the OLE1 fragment) and was used to transform the heterozygous strain ΔO8.2 (OLE1/ole1Δ::hisG).

**Overexpression of OLE1 and OLE2.** To overexpress OLE1 in _C. albicans_ we used pB1-1, a derivative of pRC2312 (Stoldt et al., 1997), containing the PCK1 promoter. The entire OLE1 coding region was amplified by genomic PCR using primers OLEHindIII-BHATG (5′-CTTTAGCTGATGCCATG-3′) and OleSpBHI-HindIII (5′-CTTTAGCTGGATCCACTTTGG-3′) (italics: HindIII, BamHI sites) and cloned into pUC18 to generate pSKM19. The 1,072 kb BamHI fragment of pSKM19 was inserted downstream of PCK1p into the BglII site of pB1-1, thereby generating OLE1-overexpression plasmid pSKM24. Similarly, the entire OLE2 ORF was amplified by genomic PCR using primers 707 and 708 (5′-TATGGATCCAGGATAATAATATG-3′/5′-TATGGATCCA-TAATCTGTAAATG-3′; italics, BamHI) and inserted, as a 1.58 kb fragment, into the BglII site of pB1-1 to generate the OLE2-overexpression vector pAP5.

For C-terminal tagging of OLE1 with green fluorescent protein (GFP), we generated a PCR fragment containing PCK1p-OLE1 with primers PCKp-HIII (5′-AGAAAGCTTGCTGAGCTCGAC-3′) and Ole1-GFP (5′-AATTTCCAGTTCGATTTTCTTCTTCC-3′) (bold, regions of homology; underlined, HindIII), using pSKM24 as a template and the resulting PCR fragment was cloned into pUC18 (pSKM58). The HindIII fragment of pSKM58 containing PCK1p-OLE1 without the stop codon was cloned into the single HindIII site in-frame with GFP into vector pRCGFP3 to generate pSKM62. To C-terminally tag OLE1 with a myc epitope, we used primers PCKp-HIII-BH-Sall (5′-GTCAGACGATCCGTTGCTGAGCGTCAC-3′) and Ole1-Myc (5′-AAATAGCTCCACGATTTTCTTCTTCC-3′) (bold, regions of homology; underlined, SalI, BamHI, HindIII) and Ole1-Myc (5′-AAATAGCTCCACGATTTTCTTCTTCC-3′) (bold, regions of homology; underlined, BamHI, italics, myc epitope) to generate a PCR fragment, which was cloned into pUC18. The BamHI fragment of the resulting plasmid pSKM59 was cloned into the BglII site of pB1-1 to generate plasmid pSKM63.

**Blotting procedures.** Total RNA was isolated from liquid cultures as described (Stoldt et al., 1997). The conditional strain ΔO7.2/25-2 was grown in SD medium without or with 0.25 mM methionine/cysteine. Following denaturing gel electrophoresis, RNA blots were probed with the 966 bp HindIII–EcoRI fragment of pSKM19, which corresponds with the 5′ region of the OLE1 ORF, or with the 938 bp EcoRI fragment derived from the OLE2 ORF.

**Immunoblotting** for detection of Ole1p-myc fusions in crude extracts of transformants carrying pSKM63 were carried out as described (Weber et al., 2001).

**Fatty acid analyses.** Lipids were extracted from cells disrupted by glass beads, as described (Daum et al., 1999), and subjected to methanolysis using BF₃/methanol. Fatty acyl methyl esters were separated by GC on a Shimadzu GC-17A (version 3) gas chromatograph using a FS-CW-20M/0-25 polarity capillary column (0.25 mm × 25 m; film thickness 0.25 mm) with a temperature gradient (160–200 °C at 1 °C min⁻¹; hold at 200 °C for 20 min). Fatty acids were identified by comparisons to a commercial standard (RM-6; Supelco).

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**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Genotype or description</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td>SC5314</td>
<td>Prototroph</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CAF2-1</td>
<td>URA3::ura3A::URE3</td>
<td>Fonzi &amp; Irwin (1993)</td>
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<tr>
<td>CA4</td>
<td>ura3A::imm434/ura3A::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
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<td>ΔO7 and ΔO8</td>
<td>As CA4, but OLE1/ole1Δ::hisG URA3 hisG</td>
<td>This work</td>
</tr>
<tr>
<td>ΔO7.2 and ΔO8.2</td>
<td>As CA4, but OLE1/ole1Δ::hisG</td>
<td>This work</td>
</tr>
<tr>
<td>ΔO7.2/25-2 and ΔO8.2/25-2</td>
<td>As CA4, but MET3p-OLE1/ole1Δ::hisG</td>
<td>This work</td>
</tr>
<tr>
<td>cI and cIV</td>
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<td>This work</td>
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<tr>
<td>h and 8.4</td>
<td>As CA4, but OLE2/ole2Δ::hisG</td>
<td>This work</td>
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<tr>
<td>JA1 and JA2</td>
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<td>This work</td>
</tr>
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<td>This work</td>
</tr>
<tr>
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<td>As CA4, but MET3p SEC20/ole1Δ::hisG</td>
<td>Weber et al. (2001)</td>
</tr>
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<td><strong>C. albicans plasmids</strong></td>
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<tr>
<td>pAP5</td>
<td>PCK1p-OLE2 in pBI-1</td>
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</tr>
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</table>
**Determination of membrane fluidity.** Cells grown to an OD$_{600}$ value of 1 (or OD$_{600}$ 0.7 for the conditional strain) were washed and converted to spheroplasts by zymolase 20T treatment. Spheroplasts were washed thrice in 20 mM Tris/HCl pH 7.5/10 mM MgSO$_4$/0.6 M sorbitol and resuspended in this buffer. An aqueous solution of 1,6-diphenyl-1,3,5-hexatriene (DPH) was prepared by diluting a 2 mM solution in tetrahydrofuran into 50 ml of 20 mM Tris/HCl pH 7.5 and removing traces of tetrahydrofuran by flushing with nitrogen. DPH (2 μM) was added to spheroplasts (4 × 10$^8$ cells ml$^{-1}$) and incubated at 30°C in a water bath shaker for 1 h. Fluorescence polarization was determined by excitation with vertically polarized monochromatic light (360 nm) and measurements of emission intensities at 426 nm using an analyser oriented parallel or perpendicular to the excitation light (Smriti et al., 1999; Kaur & Bachhawat, 1999).

The degree of fluorescence polarization (P) was calculated according to the following formula

$$P = \frac{I_{VV} - (I_{VH} \times G)}{I_{VV} + (I_{VH} \times G)}$$

where $I_V$ is the corrected fluorescence intensity and subscripts V and H indicate the values obtained with vertical or horizontal orientation, respectively, of the polarizer and analyser (in that order). The corrected fluorescence was determined by subtracting the intensity of light measured with unlabelled control spheroplasts from the intensity observed with labelled cells. The optical components used in the instruments have particular polarizing properties causing interferences which are corrected by calculating factor $G$ (called grating factor). $G$ is calculated as $I_{HH}/I_{VH}$.

**GFP fluorescence microscopy.** Cells were used for fluorescence microscopy directly without fixation. Nuclei were stained by the addition of 10 μg 4′,6-diamidino-2-phenylindole (DAPI) ml$^{-1}$ to the cell suspension. All cells were viewed using a Zeiss Axioplan 2 fluorescence microscope. Images were taken with a Quantix Digital CCD camera using METAMORPH software and processed in Corel PHOTOPAINT 11.0.

**RESULTS**

**Identification of the C. albicans OLE1 and OLE2 genes**

Inspection of C. albicans genomic sequences (http://www-sequence.stanford.edu/group/candida) revealed two genes with homology to the S. cerevisiae OLE1 gene encoding Δ9 stearoyl-CoA desaturase, which were designated OLE1 (orf6.6333; CA3921) and OLE2 (orf6.5882; CA3576). The respective gene products share 33% identity among themselves and have 57 and 32% identity, respectively, to Ole1p of S. cerevisiae (Stukey et al., 1989). The conceptual C. albicans Ole1 protein contains 486 residues, with a predicted molecular mass of 55·3 kDa, while Ole2p contains 526 residues (61 kDa). The molecular mass of Ole1p was confirmed by a myc-tagged derivative of Ole1p, which in immunoblottings of extracts of pSKM63 transformants revealed a single protein of about 55 kDa using an anti-myc antibody (data not shown). Calculation of a phylogenetic tree of fatty acid desaturases revealed close homologies among most fungal Ole1 proteins, while the C. albicans Ole2 protein, as well as the Aspergillus fumigatus Ole1 protein, were more distantly related, being situated on a common branch with mammalian desaturases (Fig. 1A).

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**Fig. 1.** Comparisons of Δ9 fatty acid desaturases. (A) A phylogenetic tree was calculated from a CLUSTAL_X alignment using the TREEVIEW software (version 1.6.5). Bar, 0-1 amino acid substitutions per site. Ole1-type proteins of Saccharomyces cerevisiae (Sc), Schizosaccharomyces pombe (Sp), Histoplasma capsulatum (Hc), Aspergillus fumigatus (Af), Homo sapiens (Hs) and Rattus norvegicus (Rn) were compared. (B) Homology among fatty acid desaturase signature regions. The fatty acid desaturase signature V sequence is underlined. Identical residues are shaded grey and highly conserved residues are marked with an asterisk.
C. albicans Ole1p contains blocks of homology compared to desaturase family 1 members, such as signature I sequences between residues 93–110 and 139–159, as well as signature V sequences between residues 309 and 323 (Fig. 1B). As with other fungal, but not mammalian, desaturases, Ole1p and Ole2p contain integral cytochrome b5 domains between residues 407–440 and residues 438–467, respectively. Computer analysis using the TMPRED program (http://www.ch.embnet.org/software/TMPRED_form.html) predicts at least four transmembrane regions, consistent with the current model for the topology of ScOle1p (Stukey et al., 1990).

**Disruption of OLE1 and OLE2 alleles**

Derivatives of the wild-type strain CAI4 were constructed which contained different disrupted OLE1 alleles (Fig. 2A). Heterozygous OLE1/ole1 strains such as strain ΔO8 were generated without difficulty, while the construction of a homozygous ole1/ole1 strain failed repeatedly. This result suggested that OLE1 in C. albicans is essential, as is ScOLE1 in S. cerevisiae (Stukey et al., 1989). To confirm this hypothesis we modified the remaining intact copy of OLE1 in the heterozygous strain ΔO8-2 by placing its ORF under transcriptional control of the MET3 promoter, which is repressed by methionine and/or cysteine (resulting

![Diagram](Fig. 2. OLE1 alleles and transcripts. (A) Top, genomic configuration of the C. albicans OLE1 wild-type locus and its deleted derivatives. H, HindIII; E, EcoRV; asterisks indicate the probe used in the corresponding Southern blot. Genomic DNA of strains was cut by HindIII and analysed by Southern blotting (bottom). Strains tested were: lane 1, CAI4 (OLE1/OLE1); lane 2, ΔO8 (OLE1/ole1Δ::hisG-URA3-hisG); lane 3, ΔO8-2 (OLE1/ole1Δ::hisG); and lanes 4 and 5, ΔO8-2/25-2 (MET3p::OLE1/ole1Δ::hisG). (B) OLE1 transcripts. Total RNA of strain CAF2-1 (lanes 1 and 4) and of the conditional strain ΔO7.2/25-2 (lanes 2, 3 and 5, 6) was analysed by Northern blotting, using a probe homologous to the OLE1 ORF. Strains were grown in the absence (lanes 1–3) and in the presence (lanes 4–6) of 0.25 mM methionine/cysteine. The positions of the MET3p-OLE1 and OLE1 transcripts, as well as of the truncated Δole1 transcript, are indicated. rRNA stained by ethidium bromide was used as loading control.)
strains ΔOLE1/OLE2. The genomic configuration of two independently constructed lineages of homozygous, heterozygous and conditional strains was verified by Southern blotting as exemplified in Fig. 2(A). Pairs of isogenic mutant strains were identical in all phenotypes, as described below.

To confirm that OLE1 in strain ΔOLE1/OLE2 was under transcriptional control of the MET3 promoter, we performed a Northern analysis of total RNA of this strain, grown without or with limiting amounts (0·25 mM) of methionine (see below). In the absence of methionine an OLE1 transcript was detected (Fig. 2B, lanes 2, 3), which was missing in the presence of methionine (lanes 5, 6). The size of this transcript of 2 kb corresponded to the OLE1 transcript in a control strain (lanes 1, 4). Growth in the presence of methionine did not influence the authentic OLE1 transcript and, in agreement, also did not regulate the expected shortened ole1Δ transcript of about 1·8 kb in the conditional strain, which arose by chromosomal integration of the MET3p-OLE1 plasmid (Care et al., 1999).

Using the above-mentioned procedure we also generated two independent lines of CAH4 derivatives, in which one or both OLE2 alleles were disrupted (data not shown). At the OLE2 locus strains CII and CIV are heterozygous (ole2/OLE2), while strains JA1 and JA2 are homozygous mutants (ole2/ole2). URA3-minus derivatives of strain JA1 were isolated by FOA selection (resulting strains JA1x and JA2x), which subsequently were transformed by the expression vector pAP5 to reconstitute OLE2. In Northern blotting using an OLE2 probe on total RNA of strain CAF2-1, we observed that OLE2 was expressed, generating a transcript of about 2 kb, but signal intensity was much lower compared to the OLE1 transcript (data not shown). Thus, OLE2 appears to be expressed at low levels, at least in the conditions used here.

Growth depends on OLE1 expression levels

The conditional strain ΔOLE1/OLE2 (MET3p-OLE1/ole1Δ::hisG) was streaked on SD medium containing cysteine and/or methionine to repress OLE1 (Fig. 3A). Growth was blocked completely at 2·5 mM cysteine, 0·25 mM methionine and 0·05 mM of a cysteine/methionine mixture, while the heterozygous strains ΔOLE1 and ΔOLE2 were not affected. Likewise, in liquid SD medium containing 2·5 mM methionine/cysteine, growth of the conditional strains was blocked completely, with a terminal phenotype of mostly unbudded cells that tended to aggregate (data not shown), suggesting a block in the G1 or G0 phase of the cell cycle. The threshold level, at which methionine/cysteine blocked growth, strongly depended on the type of media and growth conditions used: whereas cysteine/methionine at a concentration of 0·05 mM prevented growth on solid SD medium, it did not block growth in liquid SD medium up to 0·25 mM cysteine/methionine. Also, on Lee’s medium, growth of the conditional strain was inhibited significantly only at methionine concentrations above 5 mM. This medium dependence may be due to different efficiencies of MET3p repression in different conditions. The addition of 0·5 mM oleic acid in 1% lgalp CA-630 as solubilizer was able to restore growth of the conditional strain ΔOLE1/OLE2 in OLE1-repressing conditions (Fig. 3B). In contrast, the conditional control strain CA2d1m, which contains the essential SEC20 gene under control of MET3p (Weber et al., 2001), could not be rescued by oleic acid. Thus, these results indicate that OLE1 is essential in C. albicans and suggest that it is involved in oleic acid biosynthesis.

To further confirm the role of Ole1p as a fatty acid desaturase, we attempted to complement the S. cerevisiae ole1 mutant Y0779, which lacks this activity (Braun et al., 2002), by the C. albicans OLE1 gene. Expression vector pSKM24 contains CaOLE1 under transcriptional control of the C. albicans PCK1 promoter, the CAR51 replicator and the CaURA3 gene, which we found to be functional in the heterologous host S. cerevisiae. Transformants of strain Y0779 containing pSKM24 were able to grow on SD medium in the absence of added oleic acid, while transformants with the control vector pBI-1 did not grow (Fig. 3C). A similar result was obtained using medium containing 2% galactose as the carbon source, indicating that the heterologous C. albicans PCK1 promoter is active in S. cerevisiae in the presence of glucose. Taken together, these results strongly suggest that the C. albicans OLE1 gene encodes Δ9 stearoyl desaturase activity.

In contrast to the results obtained for OLE1, both alleles of OLE2 could be deleted without any difficulty and the resulting mutants (e.g. strain JA2) grew as well as the wild-type in all media, in the absence of oleic acid. Furthermore, the CaOLE2 expression vector pAP5 was unable to reconstitute the S. cerevisiae ole1 mutant Y0779. Thus, these experiments provided no evidence for a function of the OLE2 gene product as a Δ9 stearoyl desaturase.

Hyphal morphogenesis depends on OLE1 expression levels

We examined, next, if partial repression of OLE1 expression in the conditional strain would still allow growth, but prevent hyphal morphogenesis. Common media used for induction of hypha formation already contain methionine and/or cysteine: Lee’s medium contains 0·075 mM methionine (Lee et al., 1975), which in SD medium blocks growth of the conditional strain ΔOLE1/OLE2 completely (Fig. 3A); Spider and serum media contain complex sources of nitrogen likely to include methionine or cysteine. As stated above, growth of the conditional strain was not affected on Spider medium or on Lee’s medium compared to the wild-type strain. In contrast, hypha formation on both media was completely blocked (Fig. 4) and microscopy revealed that colonies consisted entirely of yeast-form cells. The importance of a sufficient dosage of wild-type OLE1 expression was confirmed by the
OLE1/ole1 heterozygous strain, which had a reduced ability to form hyphae. A more complex phenotype was observed on serum medium, on which the conditional and the heterozygous strains produced hyphae, although their growth was retarded (presumably because of methionine/cysteine as well as traces of oleic acid in serum). The effect of lowered OLE1 expression on hyphal morphogenesis was confirmed in liquid SD medium containing 5% serum and methionine/cysteine, although these experiments were hampered by the fact that both amino acids impaired hypha formation partially even in the control strain CAF2-1 (data not shown).

Fig. 3. Growth of the C. albicans ole1 conditional mutants. (A) Six independent isolates of conditional ole1 mutants (MET3p::OLE1/ole1Δ::hisG) were tested for growth at 30 °C on SD medium containing the indicated concentrations of cysteine and methionine (sectors 1–6); the heterozygous strains ΔO7 and ΔO8 (OLE1/ole1Δ::hisG-URA3-hisG) were used as controls. (B) Oleic acid complementation of the conditional ole1 mutant. Isolates of mutant ΔO8.2/25-2 (sectors 1–3) were grown on SD medium containing 1% Igepal, 2.5 mM methionine/cysteine (Met/Cys) and 0.5 mM oleic acid as indicated. As controls the wild-type CAF2-1 (OLE1/OLE1), the heterozygous strain ΔO8 (OLE1/ole1Δ::hisG-URA3-hisG) and the conditional mutant CA2d1m (MET3p-SEC20/ole1Δ::hisG) were tested. (C) Complementation of the ole1 mutation of S. cerevisiae. Transformants of the S. cerevisiae strain Y779 (ole1) carrying an empty vector (pBI-1), a vector expressing PCK1p-OLE1 (pSKM24) or PCK1p-OLE1-GFP (pSKM62) were streaked out on SD medium lacking uracil with or without 0.2% oleic acid (OA) and incubated for 3 days at 30 °C.
The addition of oleic acid to Spider medium restored hyphal morphogenesis of the conditional strain (Fig. 4), suggesting that the lack of oleic acid was the reason for the defective morphogenetic phenotype. Surprisingly, hyphal morphogenesis was also partially restored in microaerophilic conditions, in which an alternative signalling pathway leading to hyphal morphogenesis is activated (Brown et al., 1999; Sonneborn et al., 1999). This result suggested that lowered oleic acid biosynthesis does not change the ability to form hypha per se, but rather affects the induction of hypha formation.

Since these experiments had shown that low oleic acid levels could prevent morphogenesis, we also asked if increased levels would enhance hypha formation. Therefore, we grew strain CAI4(pSKM24) in inducing SCAA medium and as control cells carrying empty vector pBI-1 (data not shown). Germ tubes were of identical lengths in all strains, suggesting that different Ole1p enzyme levels were not correlated with induction and elongation of hyphae. To confirm that the growth conditions had indeed led to an overproduction of Ole1p, we grew transformant CAI4(pSKM63), producing a myc-tagged version of Ole1p, in identical conditions, and by immunoblottings could verify that Ole1p production was increased in SCAA-grown cells five- to tenfold, as compared to S4D-grown cells (data not shown).

Thus, these results indicated that a minimal dosage of Ole1p and of oleic acid is crucial to allow efficient hypha formation in several but not all conditions known to induce hyphae in C. albicans. In contrast to these results, an ole2 deletion strain (JA2) was able to form true hyphae as was the wild-type in all conditions tested, suggesting that Ole2p does not have an essential role in hyphal morphogenesis.

Chlamydospore formation requires wild-type levels of OLE1 expression

Because of the requirement for OLE1 in hypha formation, we also considered the possibility that another morphogenetic event in C. albicans, chlamydospore formation, would be affected by OLE1 expression. Therefore, we streaked the conditional MET3p-OLE1 strain onto CMA containing a low level of methionine/cysteine and covered cells by a coverslip to generate microaerophilic conditions (Joshi et al., 1993; Sonneborn et al., 1999). Following 5 days incubation at 25 °C, chlamydomspores of all strains were visible in medium lacking methionine/cysteine, while only the wild-type and the heterozygous strain produced chlamydospores in the presence of methionine/cysteine (Fig. 5). The conditional strain grew equally as well in this condition as the wild-type and heterozygous strains, but failed to form chlamydomspores and this defect remained even after prolonged incubations. In addition to this defect, the conditional strain formed pseudohyphae that aggregated strongly, which was a phenotype not seen in the heterozygous and wild-type strains. Thus, wild-type expression levels of OLE1 and corresponding levels of oleic acid are necessary to allow chlamydospore formation in C. albicans. In contrast to these results, strains JA1 and JA2 were able to form chlamydospores at normal levels, indicating that Ole2p is not involved in chlamydospore formation.

Intracellular localization of Ole1p

To examine the intracellular location of Ole1p, we examined a transformant carrying pSKM62, which expresses a fusion of OLE1 to the GFP gene under transcriptional control of the PCK1 promoter. Complementation of a S. cerevisiae ole1 mutant by pSKM62-encoded Ole1-GFP demonstrated that this fusion is a functional stearoyl CoA desaturase (Fig. 3C). In transformants grown in PCK1p-inducing medium, green fluorescence was observed throughout the
cell, but it was especially seen at a location surrounding the nucleus marked by DAPI staining (Fig. 6). This staining pattern is consistent with the localization of Ole1p in the ER membrane and agrees with the localization of the homologous protein in *S. cerevisiae* (Stukey et al., 1990). However, because at low expression levels (i.e. in S4D medium) the transformants did not show any fluorescence, we cannot exclude the possibility that high levels of Ole1-GFP biosynthesis had an influence on its intracellular distribution.

**Fatty acid analyses**

To prove the effects of altered *OLE1* or *OLE2* expression, we determined fatty acid compositions of the conditional *MET3p-OLE2* strain, the *ole2* mutant and of transformants carrying overexpression vectors. In pre-tests we observed that fatty acid compositions were strongly dependent on growth media used, in agreement with a previous study on *S. cerevisiae* (Chatterjee et al., 2001). For example, growth in SD medium led to a strong increase in C16:0 and a strong decrease in C18:2 and C18:3 fatty acids compared to growth in SCAA or SD/methionine/cysteine medium. Therefore, to exclude medium effects, we compared mutant or overexpression strains following growth in the same medium.

The conditional mutant ΔO7.2/25-2 and the control strain CAF2-1 were pre-grown in SD medium and then inoculated into SD medium containing low amounts of methionine and cysteine (0-25 mM). At these levels, the conditional strain downregulated *OLE1* to an extent to prevent hypha formation, but to still allow growth. Following about three cell doublings, we isolated lipids and generated fatty acid methyl esters, which were analysed by GC. Representative results indicate that levels of C18:1 (oleic) acids were reduced in the conditional strain, while the C18:0 precursor was strongly increased relative to amounts in the control strain (Table 2). On the other hand, levels of C18:2 and C18:3 acids were identical in the control and conditional strains, suggesting that lowering of *OLE1* expression primarily affects the C18:0 to C18:1 conversion. We also note that the levels of C16:0 and C16:1 acids were diminished in the conditional strain. In contrast, fatty acid composition in the *ole2* deletion strain JA2 appeared similar to the control strain CAF2-1 (Table 2).

We also checked if strains carrying overexpression vectors for *OLE1* or *OLE2* would show altered fatty acid compositions compared to a control strain transformed with an empty vector. A transformant with the *OLE1*-overexpression vector pSKM24 showed only a slight increase in C18:1 levels compared to the control, while contents of C18:2 and C18:3 fatty acids were not altered (Table 2). A similar pattern including an increase in C18:1 was obtained in a strain carrying the *OLE2*-overexpression vector pAP5. The latter result is the only evidence that...
OLE2, at least at elevated expression levels, may function as a Δ9 stearoyl desaturase.

**Membrane fluidity measurements**

It appeared possible that lowering of membrane oleic acid would lead to decreased membrane fluidity. Alternatively, we speculated that cells would cope with alterations in oleic acid levels by compensatory alterations in membrane lipids and/or proteins, which would maintain membrane fluidity at a relatively constant level. To decide between these alternatives, we measured membrane fluidity in strains with altered OLE1 expression levels, by detection of the mobility of the fluorochrome 1,6-diphenyl-1,3,5-hexatriene, using fluorescence polarization measurements. Results were expressed as P-values, which at low values indicate high fluorochrome mobility, i.e. high fluidity, whereas elevated values indicate decreased membrane fluidity.

Partial repression of MET3p-OLE1 in the conditional strain ΔO7.2/25-2 led to increased P-values, indicating a decrease in fluidity, as expected for a decrease in oleic acid (Table 2). On the other hand, deletion of OLE2 also decreased fluidity, although levels of unsaturated fatty acids were very similar in the ole2 mutant and the control strain. Furthermore, membrane fluidity was unaffected in a strain with an OLE2-overexpression vector and fluidity was lowered rather than increased in a transformant carrying an OLE1-overexpression plasmid relative to the control. Thus, we did not detect any correlation between fatty acid composition and membrane fluidity. A complicating factor in these studies was the fact that membrane fluidity depended on the type of growth medium, because CAF2-1 control cells grown in SCAA and SD medium had different fluidities (P-values of 0.145 and 0.142, respectively); furthermore, membrane fluidity in the control CAI4-transformant was unusually low (P = 0.184).

Since strains with different levels of overall membrane fluidity were obtained, we tested a possible correlation between membrane fluidity and the ability to form hyphae. In spite of the significantly enhanced membrane rigidity of the OLE1-overexpression strain, it was able, upon addition of 10% serum, to form hyphae with about equal kinetics compared to control cells. The different rigidities of the control strains grown in SCAA medium or SD medium also did not alter the ability of cells to form hyphae. Furthermore, the addition of the fluidizer benzyl alcohol (Horvath et al., 1998) to wild-type cells did not change the response to hypha-inducing agents (data not shown). Thus, overall membrane fluidity per se does not appear to be a crucial factor to establish morphogenetic competence, whereas levels of individual membrane components such as oleic acid, which may occur in membrane subdomains or ‘rafts’, may be relevant.

**DISCUSSION**

In this study, we characterized a first gene determining fatty acid metabolism in the human fungal pathogen *C. albicans*. The conclusion that OLE1 encodes a stearoyl desaturase required for the biosynthesis of oleic acid (C18:1) is based on (a) its high homology to such desaturases in other organisms and especially in *S. cerevisiae*, (b) its ability to complement an ole1 mutation in *S. cerevisiae*, (c) rescue of growth and morphogenetic phenotypes occurring during low OLE1 expression by external oleic acid, and (d) higher levels of oleic acid in an OLE1 overexpression strain and lowered levels in a conditional strain, in which OLE1 was downregulated. In the latter experiment, levels of C18:2 and C18:3 fatty acids, which do not occur in *S. cerevisiae*, were not downregulated, suggesting that C18:1 produced by OLE1 is not the direct precursor of C18:2 or C18:3 acids, but that a separate desaturase is involved. We suspected that the OLE2 gene described here would fulfil this function. The OLE2 gene product shares relatively low homology with OLE1 of yeast-like fungi, but has greater homology to genes encoding desaturases of filamentous fungi and mammals. However, in an ole2 deletion strain the pattern of fatty acids was similar to a wild-type strain, ruling out a function of Ole2p in the generation of C18:1, C18:2 and C18:3 fatty acids. Interestingly, a strain overexpressing OLE2 showed elevated oleic acid levels, which we take as a hint of a possible desaturase function of Ole2p, which is

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>Percentage of fatty acids</th>
<th>Membrane fluidity (P-value)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>C18:0</td>
<td>C16:0</td>
</tr>
<tr>
<td>CAF2-1</td>
<td>SD + M/C*</td>
<td>0</td>
<td>28:2</td>
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<tr>
<td>ΔO7.2/25-2 (MET3p-OLE1)</td>
<td>SD + M/C*</td>
<td>1·4</td>
<td>19:8</td>
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<tr>
<td>CAF2-1</td>
<td>SCAA</td>
<td>0</td>
<td>18:1</td>
</tr>
<tr>
<td>JAI (Δole2)</td>
<td>SCAA</td>
<td>0·7</td>
<td>19:5</td>
</tr>
<tr>
<td>CAI4(pBI-1)</td>
<td>SCAA</td>
<td>0·7</td>
<td>17:6</td>
</tr>
<tr>
<td>CAI4(pSKM24) (PCK1p-OLE1)</td>
<td>SCAA</td>
<td>0·8</td>
<td>18:7</td>
</tr>
<tr>
<td>CAI4(pAP5) (PCK1p-OLE2)</td>
<td>SCAA</td>
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</tbody>
</table>

*SD medium containing methionine and cysteine (0·25 mM).
detectable at high production levels. It is possible that OLE2 is involved in the synthesis of other fatty acid derivatives, such as leukotrienes and prostaglandins, in C. albicans (Novert et al., 2002). The task of generation of C_{18:2} and C_{18:3} fatty acids may be assumed by the gene products of two ORFs (orf6.1443; orf6.5913) in the C. albicans genome, which encode proteins that are highly homologous to Δ12 fatty acid desaturases of Aspergillus nidulans (Calvo et al., 2001).

Complete repression of OLE1 prevented growth, indicating that Ole1p and its product oleic acid provide essential functions. We presume that oleic acid is also needed for specific functions, such as for oleic acid-dependent membrane sensors, and that it has a role in regulating overall and localized membrane fluidity. Low levels of OLE1 expression did not impair growth of the yeast form, but significantly blocked hyphal morphogenesis on solid and in liquid induction media. A specific set of signalling pathways leading to hyphal morphogenesis is known to be required in most induction conditions in the presence of oxygen (Ernst, 2000), while in embedded hypoxic conditions an alternative signalling pathway, which is down-regulated in wild-type cells, is operative (Brown et al., 1999; Sonneborn et al., 1999). Because low OLE1 expression permitted hypha formation in embedded/hypoxic conditions but prevented filamentation in aerobic conditions, it appears likely that threshold levels of oleic acid are required specifically for the function of the aerobic pathways. Thus, oleic acid does not appear to have a general role in filament formation, for example for late events in hyphal development, but it is implicated in early events of activation of specific signalling pathways. Conceivably, because the biosynthesis of oleic acid requires oxygen, it could be a signalling molecule activating (yet unknown) membrane sensors transmitting external cues to internal pathways operative in aerobic conditions.

It is known that elevated temperatures increase membrane fluidity, while lowered temperatures decrease fluidity. Because elevated temperatures are a decisive environmental cue to trigger hyphal morphogenesis in C. albicans, we speculated that the state of its membrane fluidity could act as a cellular ‘thermometer’ signalling directly to morphogenetic pathways. It has indeed been reported that stress responses and in particular the heat-shock responses in yeasts are activated by membrane perturbations (Carratu et al., 1996; Moskvina et al., 1999). Lowering OLE1 expression in C. albicans indeed led to decreased membrane fluidity, as expected for a direct role of membrane fluidity in adjusting the set point of temperature induction of hyphal growth. However, further findings provide arguments against a direct correlation between membrane fluidity and morphogenesis: (1) elevated temperatures did not restore hypha formation in the MET3p-OLE1 conditional strain, (2) no correlation between membrane fluidity and hyphal induction was detected, (3) benzyl alcohol, a membrane fluidizer, did not increase hypha formation, and (4) overexpression of OLE1 led to a slightly increased level of oleic acid, but strongly increased membrane rigidity, which nevertheless did not interfere with hypha formation. Increased rigidity in the latter experiments may be due to compensatory increases in other membrane components, for example an increase in ergosterol levels, which may maintain the physical state of membranes constant. According to a similar principle, it has been described that the composition of the C. albicans cell wall is subject to compensatory alterations (Kapteyn et al., 2000). Furthermore, a genome-wide transcriptional profiling of genes induced during hyphae induction recently revealed that a heat shock alone is not able to induce hypha-specific genes and hyphal morphogenesis (Nantel et al., 2002). Thus, we favour a model in which stress responses and induction of morphogenesis do not share the same dependence on membrane fluidity. We rather postulate that levels of oleic acid have a direct effect on specific components of the hyphal induction machinery.

In addition to the defect in hyphal morphogenesis, the development of chlamydospores was blocked at low OLE1 expression levels. Although the functions of the thick-walled chlamydospores in the biology and virulence of C. albicans are currently unclear, it is evident that their development requires a specific morphogenetic pathway, which depends on oleic acid. The finding of a threshold level of oleic acid for morphogenetic events in C. albicans suggests that the reason for defective phenotypes in several morphogenetic mutants of C. albicans may be an impaired lipid and/or fatty acid and/or oleic acid metabolism. In agreement with this notion, in S. cerevisiae the Tup1 regulator represses transcription of OLE1 (Fujimori et al., 1997) and C. albicans tup1 mutants grow in a pseudohyphal form (Braun & Johnson, 1997), while we observed that OLE1 overexpression favours an abnormal pseudohyphal growth form, although only in a small fraction of cells. Our results further suggest that Ole1p may be a suitable target for future antifungal agents. Because OLE1 is essential, it is likely that potential Ole1 inhibitors will prevent cell growth and lead to a rapid loss of viability. This would be an advantage compared toazole inhibitors of ergosterol biosynthesis, which do not kill fungal pathogens. Even at low doses of inhibitors, which reduce but do not eliminate Ole1 function, hyphal morphogenesis and consequently virulence of C. albicans would be blocked. A major structural difference between mammalian and fungal Ole1 proteins is the presence of an integral cytochrome b_{5} domain in fungal desaturases, which may allow the development of selective inhibitors.

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