A third fatty acid $\Delta 9$ -desaturase from *Mortierella alpina* with a different substrate specificity to ole1p and ole2p

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A third gene ($\Delta 9$ -3) encoding a fatty acid $\Delta 9$ -desaturase was isolated from the oil-producing fungus *Mortierella alpina*. The predicted protein of 512 aa shared 53% sequence identity with the two fatty acid $\Delta 9$ -desaturases, ole1p and ole2p, already described in this organism and contained three histidine boxes, four putative transmembrane domains and a C-terminal cytochrome *b₅* fusion that are typical of most fungal membrane-bound fatty acid desaturases. However, unlike the *M. alpina* ole1 and ole2 genes, the $\Delta 9$ -3 ORF failed to complement the *Saccharomyces cerevisiae* ole1 mutation. GC-MS analysis of fatty-acid-supplemented ole1 yeast transformants containing the $\Delta 9$ -3 gene indicated that this enzyme had negligible activity with endogenous palmitic acid (16:0) as substrate and moderate activity (30–65% desaturation) with endogenous stearic acid (18:0). Yeast transformants overexpressing any one of the three *M. alpina* fatty acid $\Delta 9$ -desaturase genes or the *S. cerevisiae* OLE1 gene produced low amounts of hexacosenoic acid [26:1(n-9)], a fatty acid that is not normally present in yeast cells. It follows that these $\Delta 9$ -desaturases may also display low n-9 desaturation activity with very long-chain saturated fatty acid substrates. Conversely, high levels of desaturase in the endoplasmic reticulum membrane of these yeast transformants may increase the availability of suitable monounsaturated substrates for fatty acid elongation.

Keywords: stearyl-CoA desaturase, hexacosenoic acid, fatty acid n-9 desaturation, fatty acid elongation, yeast complementation

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Abbreviations: 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α -linolenic acid; γ -18:3, γ -linolenic acid; 20:0, arachidic acid; 20:1, eicosenoic acid; 20:3, dihomogamma-linolenic acid; 20:4, arachidonic acid; 22:0, behenic acid; 22:1, erucic acid; 24:0, lignoceric acid; 24:1, nervonic acid; 26:0, hexacosanoic acid; 26:1, hexacosenoic acid; $\Delta 9$ -3, gene encoding fatty acid $\Delta 9$ -desaturase homologue; DMOX, 4,4-dimethyloxazoline; ELO2 and ELO3, genes encoding fatty acid elongases; ER, endoplasmic reticulum; FAME, fatty acid methyl ester; LCPUFA, long-chain polyunsaturated fatty acid; ole1 and ole2, genes encoding fatty acid $\Delta 9$ -desaturases ole1p and ole2p, respectively; RACE, rapid amplification of cDNA ends; UTR, untranslated region of mRNA.

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INTRODUCTION

In filamentous fungi, the pathway for fatty acid desaturation and elongation from stearic acid (18:0) to long-chain polyunsaturated fatty acids (LCPUFAs) has been elucidated both by biochemical means and by studying mutant strains isolated via classical mutagenesis (Ratledge, 1993; Certik *et al.*, 1998). The substrate for the initial $\Delta 9$ -desaturation of 18:0 to oleic acid (18:1) is stearyl-CoA, but some of the subsequent desaturation steps, including $\Delta 12$ - and $\Delta 6$ -desaturation, occur using the fatty acyl chains of phospholipid molecules (Jackson *et al.*, 1998). In the oil-producing fungus *Mortierella alpina* most of the genes encoding the fatty acid desaturases and elongases of the LCPUFA

biosynthetic pathways have been isolated and characterized (Michaelson *et al.*, 1998; Huang *et al.*, 1999; Sakuradani *et al.*, 1999; Wongwathanarat *et al.*, 1999; Parker-Barnes *et al.*, 2000). Interestingly, two fatty acid $\Delta 9$ -desaturase genes were isolated from this fungus (Wongwathanarat *et al.*, 1999) and these have been shown to be expressed differentially in response to growth temperature and nutrient levels (A. T. Carter, unpublished results).

Multiple fatty acid $\Delta 9$ -desaturases, encoded by distinct genes, have been found in a number of other organisms, including rat (Thiede *et al.*, 1986; EMBL accession no. AB032243), mouse (Tabor *et al.*, 1998; Zheng *et al.*, 2001), carp (Tiku *et al.*, 1996; EMBL accession no. AJ249259), *Drosophila melanogaster* (Wicker-Thomas *et al.*, 1997), sesame (Yukawa *et al.*, 1996), *Arabidopsis thaliana* (Fukuchi-Mizutani *et al.*, 1998) and *Caenorhabditis elegans* (Watts & Browse, 2000). Although differential expression of these genes has been observed in many cases, the significance of this is not always clear. The three fatty acid $\Delta 9$ -desaturase homologues identified in *Caenorhabditis elegans* displayed different substrate specificities when expressed in yeast but their roles in *Caenorhabditis elegans* have yet to be elucidated (Watts & Browse, 2000). In carp, *CDS1* and *CDS2*, encoding two closely related fatty acid $\Delta 9$ -desaturases, responded differently to low temperature (Tiku *et al.*, 1996) and nutritional (S. Polley, M. X. Caddick & A. R. Cossins, personal communication) stimuli and exhibited tissue-specific expression. The differences in expression were thought to be part of co-ordinated, biosynthetic responses to body cooling or dietary changes. In the present paper, we describe the isolation of a gene ($\Delta 9$ -3) encoding a third fatty acid $\Delta 9$ -desaturase from *M. alpina* and its functional characterization in yeast.

METHODS

Strains, media and growth conditions. *Mortierella alpina* strain CBS 528.72 (ATCC 32222) was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. *Saccharomyces cerevisiae* strain L8-14C (MATa *ole1Δ::LEU2 leu2-3 leu2-112 ura3-52 his4*; Stukey *et al.*, 1989) was supplied by Professor M. Schweizer, Heriot-Watt University, Edinburgh. *S. cerevisiae* strain YN99-90 (MATα *OLE1 ura3-52 trp1Δ63*) was kindly supplied by Bruce Pearson, Institute of Food Research, Norwich. *S. cerevisiae* strain YD15281 (MATα *elo3Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*; Winzeler *et al.*, 1999) was a gift from Dr A. Hayes, University of Manchester. *S. cerevisiae* strain L8e1 (MATa *elo3Δ::kanMX4 ole1Δ::LEU2 leu2-3 leu2-112 ura3-52 his4*) was constructed from L8-14C by PCR-mediated *kanMX4* gene deletion of *ELO3* as described below. *M. alpina* and *S. cerevisiae* strains were cultured as described previously (Wongwathanarat *et al.*, 1999). *M. alpina* growth medium S2lowN was a modified version of S2GYE (MacKenzie *et al.*, 2000) containing 0.01% (w/v) yeast extract (Oxoid) and 0.01% (w/v) NH_4SO_4 . In yeast feeding experiments, unsaturated fatty acids were added at a final concentration of 0.5 mM to medium containing 1% (w/v) tertitol NP-40 (Sigma) (Wongwathanarat *et al.*, 1999), whereas saturated fatty acids were first dissolved in 90% (v/v) ethanol at a concentration of 1 mM and added to medium lacking tertitol

to give a final concentration of 0.04 mM. In the latter, control cultures were supplemented with 3.6% (v/v) ethanol.

Nucleic acid manipulations. Degenerate primers with homology to conserved histidine boxes 2 and 3 of fatty acid $\Delta 9$ -desaturase genes from *S. cerevisiae* (Stukey *et al.*, 1990), *Histoplasma capsulatum* (Gargano *et al.*, 1995) and *Cryptococcus curvatus* (Meesters & Eggink, 1996) were synthesized on an ABI 394 DNA/RNA synthesizer. Primer combination P5 (5'-CAYMGVTAYACNGAYAC-3'; 192-fold degeneracy) and P2 (5'-RTGRTGRAARTTTRTGRT-3'; 64-fold degeneracy) was used to amplify a fragment (P5-P2) from *M. alpina* CBS 528.72 genomic DNA. PCR conditions were 94 °C hot start for 5 min, 30 cycles of 94 °C for 0.5 min, 52 °C for 1.5 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The P5-P2 fragment was subsequently used to screen a *Bam*HI genomic DNA library from *M. alpina* CBS 528.72 (Wongwathanarat *et al.*, 1999) and positive pBK-CMV phagemid clones (Stratagene) were excised *in vivo* and sequenced. The following primer pairs specific to the *M. alpina* $\Delta 9$ -3 gene were used to analyse intron removal from mRNA by RT-PCR (Wongwathanarat *et al.*, 1999) under standard PCR conditions at a primer annealing temperature of 52 °C: M9 (5'-TCCTCGGGCAAGAGCTCCAG-3') and M1 (5'-GCCATGAGACACACAGTCGATG-3'), and P5F (5'-ACCATCGTTACACTGACACG-3') and P2R (5'-ACTCGTGGTGGAAAGTTGTGG-3'). RT-PCR also identified the $\Delta 9$ -3 3' untranslated region (UTR) using the degenerate oligo(dT)₁₈ primer AD99 [5'-AAGCGGCCGC(T)₁₈VN-3'] in the cDNA first-strand synthesis step. Subsequent PCR with gene-specific primer D4 (5'-GAGAAGGGGCAGGCTGTGACT-3') was carried out under standard conditions at a primer annealing temperature of 50 °C. RNA-ligase-mediated rapid amplification of cDNA 5' ends (RLM-RACE), using a First-Choice RLM-RACE kit (Ambion), identified the $\Delta 9$ -3 5'-UTR. Gene-specific primers were D9R1 (5'-GCAGTGATGCCAGTCCGGTG-3') and D9R2 (5'-GGATCGCTTTGATGTGCTTGG-3'). PCRs were carried out as per the kit instructions, using a 60 °C primer annealing temperature.

The *ELO3* gene in *S. cerevisiae* L8-14C was deleted using a PCR-generated *kanMX4* cassette (Wach *et al.*, 1994; Winzeler *et al.*, 1999) with 40 bp flanking regions homologous to the *ELO3* gene. Forward primer ELO35K (5'-ATTCGGCTTTTTCCTGTTTGTACGAAACATAAACAGTCAGCTGAAGCTTCGTACGCTG-3') and reverse primer ELO33K (5'-ATTCTCGCTTCCTATTTAAGCTTTCCTGGAAGAAGACCTTGATAGGCCACTAGTGATCTG-3') were used at an annealing temperature of 54 °C to amplify the 1.6 kb *ELO3-kanMX4* cassette with vector pFA6a-*kanMX4* as template (Wach *et al.*, 1994). Approximately 1 µg of *ELO3* deletion cassette DNA was then transformed into PEG/LiAc-treated whole L8-14C cells, following the method of Gietz *et al.* (1995), and transformants were selected on 16:1/18:1-supplemented medium containing G418 (200 µg ml⁻¹). *ELO3*-deleted transformants were screened by whole-cell PCR using gene-specific forward and reverse primers ELO3cF (5'-TACCAGTTAGGTACTGTG-3') and ELO3cR (5'-CTTTGGGCAGCTAAGGAAATG-3') at an annealing temperature of 58 °C. *ELO3*-deleted strain L8e1 generated a 1.9 kb PCR fragment, while non-deleted strains gave a 1.5 kb fragment. Deletion of the *ELO3* gene in L8e1 was confirmed by analysing this strain's fatty acid composition following growth in YNB medium supplemented with 16:1/18:1.

Construction of yeast expression vectors. Before the transcriptional start point (*tsp*) of the $\Delta 9$ -3 gene was identified by 5'-RACE, a 5'-truncated version of the $\Delta 9$ -3 ORF (5'- $\Delta 9$ -3), lacking the first 21 codons, was initially cloned into the yeast

expression vector pVT100-U (Vernet *et al.*, 1987; Wongwathanarat *et al.*, 1999). A synthetic, intronless version of this truncated gene with *Bam*HI and *Xho*I sites at the 5' end and a *Bam*HI site at the 3' end was created in three steps by overlap extension PCR using a genomic clone as template. First, the following primer combinations were used to amplify three PCR products, B1–B2 (413 bp), B3–B4 (195 bp) and B5–B6 (979 bp) in which the two introns were removed: B1 (5'-AAGGATCCCTCGAGAAAAAATGAAAACGTCTCTCTCCGCT-3') and B2 (5'-AGACCACAGTTCGATGGTATC=CAGCAGTGATGCCAGTCCGGT-3'), B3 (5'-ACCGGACTGGGCATCACTGCTG=GATACCATCGACTGTGGTCT-3') and B4 (5'-CTTTTGGGCACCATAGGGTTC=CTTTTCCGTGTCAGTGTAAAC-3'), and B5 (5'-GTTACACTGACACGGAAAAAG=GACCCCTATGGTGCCCAAAG-3') and B6 (5'-AAGGATCCTTATGCGGTGCGACTCACAACAGGTATAG-3'), where the start and stop codons are single-underlined, the relevant restriction sites are double-underlined and the intron deletion sites are indicated by a double hyphen. In the second step, fragments B1–B2 and B3–B4 were fused together using primers B1 and B4 to generate a 566 bp PCR product (B1–B4). Finally, the intronless 1504 bp 5'- $\Delta 9$ -3 ORF was created by fusing fragments B1–B4 and B5–B6 in a PCR with primers B1 and B6. All PCRs were carried out as follows: 5 min hot start at 94 °C, 25 cycles of 94 °C for 0.5 min, 56 °C for 1.5 min, 72 °C for 1 min and a final 10 min extension at 72 °C. The final PCR product (B1–B6) was digested with *Xho*I and *Bam*HI and directionally cloned into pVT100-U. The sequence of the insert was checked using primers ADH1 and ADH2 (Wongwathanarat *et al.*, 1999) from the yeast *ADH1* promoter and terminator regions.

pVT100-U containing the full-length $\Delta 9$ -3 ORF was subsequently produced by replacing a 70 bp *Pst*I fragment at the 5' end of the 5'- $\Delta 9$ -3 version with a 133 bp *Pst*I fragment containing the extra 21 N-terminal codons of the complete gene. This fragment had been amplified from a $\Delta 9$ -3 genomic clone using primers 93–5P (5'-GCTGCTGCAGGCTCGAGAAAAAATGGGATCGCTCACTTCG-3') and 93–3P (5'-CACTGTCGCTGCAGTCACTGTTG-3'), where the $\Delta 9$ -3 start codon is single-underlined and the *Pst*I sites are double-underlined. The fragment was generated using *Taq*Plus Precision (Stratagene) with the following PCR conditions: 1 min hot start at 94 °C, 5 cycles of 1 min at 94 °C, 1 min at 54 °C, 20 s at 72 °C, 20 cycles of 1 min at 94 °C, 1 min at 62 °C, 20 s at 72 °C, followed by 3 min at 72 °C and 10 min at 35 °C. It was then digested with *Pst*I and ligated into purified *Pst*I-cut pVT100-U:5'- $\Delta 9$ -3. Clones containing the full-length $\Delta 9$ -3 ORF in pVT100-U were identified by PCR and the insert sequenced to verify that the gene was in the correct orientation.

The *S. cerevisiae* *OLE1* ORF was amplified from plasmid pGAL-OLE2.8 (Gonzalez & Martin, 1996) which was kindly supplied by Professor C. E. Martin, Rutgers University, Piscataway, New Jersey, USA. An *Xho*I site was introduced at the 5' end of this gene and a *Bam*HI site at the 3' end using primers 5OLE1 (5'-AACTCGAGAAAAAATGCCAATTCTGGAAC-3') and 3OLE1 (5'-AAGGATCCTTATAC-TTAAAGAACTTACCAGTTTC-3') in a *Taq*Plus Precision (Stratagene) PCR at an annealing temperature of 52 °C. PCR products were cloned into pCR4-TOPO (Invitrogen) and sequenced. The *S. cerevisiae* *OLE1* ORF was then directionally cloned as an *Xho*I–*Bam*HI fragment into pVT100-U.

Yeast transformation and fatty acid analysis. pVT100-U containing either the full-length $\Delta 9$ -3 ORF or the *S. cerevisiae* *OLE1* ORF was transformed into yeast strains by the PLATE method (Elble, 1992). Approximately 0.5 µg vector DNA, 100 µg single-stranded herring sperm DNA and one large

toothpick of freshly grown yeast cells were used per transformation. Plasmid-containing cells were selected on uracil-minus YNB agar with or without 16:1/18:1 supplementation (Wongwathanarat *et al.*, 1999). Control transformations were also carried out with pVT100-U lacking any inserts. Transformants were subsequently grown in uracil-minus YNB broth with or without fatty acid supplementation. Fatty acid methyl esters (FAMES) were prepared as described previously (Wongwathanarat *et al.*, 1999) and analysed by GC-MS. FAMES in n-hexane (2 µl) were separated on a 30 m BPX70 0.25 µm column (SGE) in a 5890 Series 2 gas chromatograph (Hewlett Packard) using an injection port temperature of 250 °C and the following column temperatures: 1 min at 110 °C, 110–240 °C at a gradient of 5 °C min⁻¹, 10 min at 240 °C. Mass spectra were produced over a scan range of 40–500 *m/z* in a TRIO-1S mass spectrometer (Fisons Instruments VG Organic) that was coupled to the gas chromatograph and operated at an ionization voltage of 70 eV, a source temperature of 200 °C and an interface temperature of 240 °C. To detect FAMES present at lower concentrations, extracts were concentrated by up to 25-fold before analysing 2 µl as described above. Fatty acid 4,4-dimethyloxazoline (DMOX) derivatives were prepared by reaction of FAMES with 2-amino-2-methyl-1-propanol at 180 °C for 18 h (Fay & Richli, 1991). Samples were dissolved in 500 µl iso-hexane and 5 µl analysed by GC-MS on a 100 m CPSil88 column (Chrompack) in a 6890 gas chromatograph (Agilent) with an injection port temperature of 270 °C. The following GC running conditions were used: 35 min at 185 °C; 185–190 °C at a gradient of 0.5 °C min⁻¹; 5 min at 190 °C; 190–220 °C at a gradient of 0.5 °C min⁻¹; 20 min at 220 °C. The system was pre-calibrated with 68D FAMES Standard (Nu-Chek Prep.). Mass spectral analysis was performed in electron ionization mode in a 5973 MS (Agilent) over a scan range of 100–550 *m/z*.

RESULTS AND DISCUSSION

Isolation of the $\Delta 9$ -3 fatty acid desaturase gene from *M. alpina*

A PCR fragment (P5–P2) of 524 bp was generated from genomic DNA of *M. alpina* CBS 528.72 using the degenerate primers P5 and P2 that anneal to sequences in conserved histidine boxes 2 and 3 of fungal fatty acid $\Delta 9$ -desaturases. This fragment contained a putative intron of 110 bp and when the ORF was translated its 138 aa product had 63 % identity to the ole1p and ole2p fatty acid desaturases from *M. alpina* (Wongwathanarat *et al.*, 1999). The P5–P2 fragment was subsequently used to screen a genomic DNA library of *M. alpina* CBS 528.72 (Wongwathanarat *et al.*, 1999) and all positive clones contained an identical 8.3 kb *Bam*HI insert that had also been identified by Southern analysis (data not shown). On sequencing, this 8288 bp insert contained a putative fatty acid desaturase gene, designated $\Delta 9$ -3, that encoded a protein of 512 aa. The $\Delta 9$ -3 ORF was interrupted by two introns of 960 and 110 nt (Fig. 1), the border sequences of which (5'-GT...AG-3') conformed to the consensus for fungal introns (Gurr *et al.*, 1987). The position of intron I in relation to the $\Delta 9$ -3 protein sequence was conserved with that of the single intron in *M. alpina* *ole1* and *ole2*, occurring immediately upstream of histidine box 1 (Wongwathanarat *et al.*, 1999).

Fig. 1. Structural organization of the $\Delta 9-3$ gene from *M. alpina*. The ORF is indicated by three open boxes with the size of the encoded protein given (aa) within each box. The three conserved histidine boxes are marked 1, 2 and 3, respectively: 1, HRLWSH; 2, HRAHH; 3, HNFHH. The haem-binding pocket of the cytochrome b_5 domain, EHPGG(X)₁₀DAT(X)₉HS, is indicated by b_5 . The position and size (nt) of the 5'- and 3'-UTRs and of the two introns are given in bold and italic text below the line (not to scale). The arrows indicate the direction and approximate positions of the primers used in this study: a, P5; b, P2; c, M9; d, M1; e, P5F; f, P2R; g, D4; h, AD99.

Fig. 2. Sequence comparison of the $\Delta 9-3$ protein with the *M. alpina* ole1p and ole2p fatty acid desaturases. The positions of the three histidine boxes are indicated by 1, 2 and 3 and that of the cytochrome b_5 haem-binding pocket by hatched bars below the sequence. The beginning of the cytochrome b_5 domain is shown by the arrow. Approximate positions of the four putative transmembrane domains are indicated by double-headed arrows.

Correct splicing of both introns in $\Delta 9-3$ was confirmed by RT-PCR analysis using primer pairs M9/M1 and P5F/P2R (Fig. 1) with RNA that was isolated from 4-day-old PDB-grown cultures. Low levels of $\Delta 9-3$ transcript, about 1.9 kb in size, could be detected by Northern analysis in *M. alpina* from day 4 onwards

under these growth conditions, but a more complete study of the transcription and regulation of this gene is currently underway (A. T. Carter, unpublished results). Interestingly, intron I, at 960 nt, is extremely long for filamentous fungi but other large introns of >300 nt have also been reported in this organism (Kobayashi *et*

Fig. 3. Unrooted radial tree of fungal and animal fatty acid Δ^9 -desaturases displayed using the TREEVIEW programme (Page, 1996). Protein sequences were aligned using CLUSTALX from the University of Wisconsin GCG software package. Ccurv, *Cryptococcus curvatus*; Hcaps, *H. capsulatum*; Ma, *M. alpina*; Mucor, *Mucor rouxii*; Pichia, *Pichia pastoris*; Scerev, *S. cerevisiae*; cefat, *Caenorhabditis elegans* (note that the enzymes from *Cryptococcus curvatus* and *H. capsulatum* are from two different strains). The scale bar represents 0.1 aa changes per site.

al., 1999; A. T. Carter, unpublished results). 5'-RACE and RT-PCR analysis indicated that the 5'- and 3'-UTRs of this gene were approximately 125 and 163 nt, respectively, and a near-consensus poly(A) addition signal (CATAAA) was present 18 nt upstream of the poly(A) addition site. In addition to the putative fatty acid desaturase gene, the 8288 bp genomic DNA fragment contained an incomplete gene (accession no. AJ249747), encoding the C-terminal part of a protein with putative ankyrin repeat sequences (Sedgwick & Smerdon, 1999), upstream of the desaturase gene and a putative heat-shock protein gene (*hsp10*; accession no. AJ249748) downstream.

The predicted Δ^9 -3 protein contained three histidine boxes, four putative transmembrane domains and a C-terminal cytochrome b_5 fusion that are typical of a fungal fatty acid desaturase (Fig. 2) (Napier *et al.*, 1999; Sperling & Heinz, 2001). This protein shared 53 % aa identity with the *M. alpina* ole1p and ole2p desaturases and 40–50 % identity with other fungal fatty acid Δ^9 -desaturases. Conversely, it had little or no homology to the other fatty acid desaturases from *M. alpina* (Michaelson *et al.*, 1998; Huang *et al.*, 1999; Sakuradani *et al.*, 1999), except for about 25 % aa identity to the N-terminal cytochrome b_5 regions of the Δ^5 - and Δ^6 -desaturases. A similar low level of amino acid identity was observed with the N-terminal cytochrome b_5 regions of a related class of enzymes, the plant sphingolipid desaturases (Sperling *et al.*, 1998; Libisch *et al.*,

2000). The Δ^9 -3 desaturase was 67 aa residues longer than *M. alpina* ole1p and ole2p, with the bulk of the difference being a 43 aa extension at the N terminus, but it was similar in size to the fatty acid Δ^9 -desaturase from *S. cerevisiae* (Stukey *et al.*, 1990). The spacings between histidine boxes and cytochrome b_5 domain in Δ^9 -3 and the amino acid sequences of these motifs were identical or very similar to those of other fungal Δ^9 -desaturases. One interesting observation was that the sequences of histidine box 1 (HRLWSH) and the cytochrome b_5 haem-binding pocket [EHPGG(X)₁₀DAT(X)₉HS] were identical to those of *S. cerevisiae* Ole1p and differed from those of *M. alpina* ole1p and ole2p [HRLWAH and EHPGG(X)₁₀DMT(X)₉HS, respectively]. On the other hand, the sequence of histidine box 2 (HRAHH) was the same as that of the other *M. alpina* Δ^9 -desaturases and differed from the yeast sequence (HRIHH). The significance of these sequence differences remains unclear, but may reflect the evolutionary divergence of Δ^9 -3 from the *M. alpina* ole1 and ole2 genes. When displayed on a phylogenetic tree, the Δ^9 -3 protein was less related than the *Mucor rouxii* ole1p to the *M. alpina* ole1p and ole2p (Fig. 3). This tree illustrates the clear distinction between animal and fungal fatty acid Δ^9 -desaturases and also shows the divergence within species that contain more than one Δ^9 -desaturase homologue in the same strain (mouse, rat, carp, *Caenorhabditis elegans* and *M. alpina*).

Functional characterization of the *M. alpina* Δ^9 -3 desaturase gene in *S. cerevisiae*

The *in vivo* function of the Δ^9 -3 gene was examined by cloning the Δ^9 -3 ORF into the yeast expression vector pVT100-U (Vernet *et al.*, 1987) and transforming this into the *S. cerevisiae* ole1 mutant L8-14C (Stukey *et al.*, 1989). Unlike the *M. alpina* ole1 and ole2 genes (Wongwathanarat *et al.*, 1999), Δ^9 -3 failed to complement the yeast ole1 mutation when URA transformants were grown without 16:1/18:1 supplementation. To investigate the activity of the Δ^9 -3 gene product further, the pVT100-U: Δ^9 -3 construct was transformed into an OLE1 wild-type yeast strain YN99-90. The fatty acid profiles of pVT100-U: Δ^9 -3 transformants and of control strains carrying empty vector were examined in detail by GC-MS and compared with previous data from ole1 yeast strains containing either the *M. alpina* ole1 or ole2 genes (Wongwathanarat *et al.*, 1999). Fig. 4(a–c) illustrates typical profiles from YN99-90 transformants grown in unsupplemented medium. These were very similar to the profiles from L8-14C transformants cultivated in 16:1/18:1-containing medium (data not shown). In addition, use was made of the ability of yeast strain L8-14C to grow in media supplemented with only a single monounsaturated fatty acid (Stukey *et al.*, 1989) or linoleic acid [18:2(n-6); McDonough *et al.*, 1992]. L8-14C transformants containing the Δ^9 -3 gene were grown in media with 16:1, 18:1 or 18:2 as sole supplement to investigate the specificity of the Δ^9 -3 enzyme with respect to 16:0 and 18:0 (Table 1).

Fig. 4. Typical gas chromatograms of FAMES prepared from *S. cerevisiae* transformants. (a) YN99-90 (*OLE1*) transformed with empty vector pVT100-U (2 μ l FAME extract); (b) YN99-90(pVT100-U) (equivalent to 50 μ l FAME extract); (c) YN99-90(pVT100-U: Δ 9-3) (equivalent to 8 μ l FAME extract); (d) YD15281 (*OLE1 elo3* Δ)(pVT100-U) (equivalent to 40 μ l FAME extract); (e) YD15281(pVT100-U: Δ 9-3) (equivalent to 12 μ l FAME extract); (f) YD15281(pVT100-U:Sc*OLE1*) (equivalent to 4 μ l FAME extract). Peaks marked with an asterisk are unidentified. All transformants were grown in unsupplemented medium.

Table 1. Fatty acid composition of *S. cerevisiae ole1* transformants containing either the *M. alpina* $\Delta 9$ -3, *ole1* or *ole2* genes

L8-14C (*ole1*) yeast transformants containing empty vector pVT100-U, pVT100-U: $\Delta 9$ -3, pVT100-U:*ole1* or pVT100-U:*ole2* were incubated at 25 °C in YNB medium with a range of fatty acid supplements. After 4 days growth, fatty acids were transmethyated directly from washed, freeze-dried cells and analysed by GC-MS. The relative amount of each fatty acid was calculated from the GC peak area and expressed as a percentage of total fatty acids ($\pm$ SD) after subtracting the peak area of the supplemented fatty acid from the total. NG, No growth.

L8-14C transformant (no. tested)	Fatty acid supplement	16:0 (%)	16:1 (%)	Desaturation 16:0 to 16:1 (%)	18:0 (%)	18:1 (%)	Desaturation 18:0 to 18:1 (%)
Empty pVT100-U (4)	None	NG	NG	NG	NG	NG	NG
	16:1	51.7 $\pm$ 2.3	—	—	35.4 $\pm$ 4.7	0	0
	18:1	76.8 $\pm$ 10.9	0	0	11.7 $\pm$ 0.4	—	—
	18:2	90.3 $\pm$ 1.8	0	0	6.8 $\pm$ 3.3	0	0
pVT100-U: $\Delta 9$ -3 (5)	None	NG	NG	NG	NG	NG	NG
	16:1	60.1 $\pm$ 9.8	—	—	17.6 $\pm$ 9.9	18.6 $\pm$ 5.4	53.2 $\pm$ 17.5
	18:1	89.4 $\pm$ 14.0	0.9 $\pm$ 1.9	1.2 $\pm$ 2.7	4.9 $\pm$ 4.9	—	—
	18:2	89.6 $\pm$ 0	0	0	2.6 $\pm$ 0.2	4.6 $\pm$ 0.2	63.4 $\pm$ 2.8
pVT100-U: <i>ole1</i> (4)	None	21.6 $\pm$ 3.8	6.8 $\pm$ 1.4	24.3 $\pm$ 1.2	1.4 $\pm$ 1.1	62.6 $\pm$ 9.3	97.7 $\pm$ 1.5
pVT100-U: <i>ole2</i> (4)	None	26.4 $\pm$ 5.3	3.9 $\pm$ 0.7	13.3 $\pm$ 2.9	2.3 $\pm$ 2.2	61.3 $\pm$ 15.7	96.0 $\pm$ 5.2
<i>OLE1</i> (4)*	None	16.4 $\pm$ 4.9	53.3 $\pm$ 10.0	76.0 $\pm$ 9.2	6.4 $\pm$ 3.5	20.7 $\pm$ 1.6	77.0 $\pm$ 9.4

* Untransformed *S. cerevisiae* strain YN99-90 (*OLE1*).

Table 2. Production of 26:1(n-9) in *S. cerevisiae* transformants containing either the *M. alpina* $\Delta 9$ -3, *ole1* or *ole2* genes

Cultures of L8-14C transformants containing empty vector pVT100-U or pVT100-U: $\Delta 9$ -3 were grown at 25 °C for 4 days in YNB medium supplemented with 16:1/18:1, whereas all other transformants were grown in unsupplemented medium. Fatty acids were transmethyated directly from washed, freeze-dried cells, concentrated 25-fold in n-hexane and analysed by GC-MS. The relative amount of each fatty acid was calculated from the GC peak area and expressed as a percentage of total fatty acids ($\pm$ SD).

Yeast strain	<i>M. alpina</i> gene	No. of transformants tested	Total fatty acids (%)	
			26:0	26:1
YN99-90 (<i>OLE1</i>)	U/E*	6	0.14 $\pm$ 0.04	0
	$\Delta 9$ -3	15	0.06 $\pm$ 0.07	0.11 $\pm$ 0.09
L8-14C (<i>ole1</i>)	U/E*	5	0.24 $\pm$ 0.14	0
	$\Delta 9$ -3	9	0.04 $\pm$ 0.02	0.24 $\pm$ 0.12
	<i>ole1</i>	4	0.05 $\pm$ 0.02	0.09 $\pm$ 0.06
	<i>ole2</i>	4	0.15 $\pm$ 0.05	0.09 $\pm$ 0.03

* Results combined for untransformed strains (U) and strains containing empty vector pVT100-U (E).

In the yeast *ole1* background, $\Delta 9$ -3 showed negligible activity with 16:0 as substrate [0–6% desaturation to 16:1(n-7)], during growth with 18:1 or 18:2 supplementation, but greater activity with 18:0 as substrate [30–65% desaturation to 18:1(n-9)], during 16:1 or 18:2 supplementation. This compared with conversion values of 10–25% 16:0 to 16:1(n-7) and 90–99% 18:0 to 18:1(n-9) for the other two *M. alpina* $\Delta 9$ -desaturases, *ole1p* and *ole2p* (Wongwathanarat *et al.*, 1999; Table 1). The position of the double bond in the 18:1 that was produced by $\Delta 9$ -3 transformants was confirmed as n-9 by DMOX derivatization (Fay & Richli, 1991). DMOX-

derivatized fatty acids give mass spectra that are more easily interpreted than those from FAMES, with a greater abundance of higher mass species, including the full-length molecular ion. Another major advantage is the reduction in double bond ionization and migration along the alkyl chain which, in most cases, facilitates the unambiguous positioning of double bond(s) in a fatty acid. The amount of 18:1(n-9) produced in $\Delta 9$ -3 transformants was low, for example, relative to the amount of endogenous 16:0. From the data presented in Table 1, it can be inferred that the total amount of monounsaturated fatty acids (16:1 plus 18:1) produced

Fig. 5. GC-MS analysis of DMOX-derivatized 26:1 produced by *S. cerevisiae ole1* mutant L8-14C containing pVT100-U:Δ9-3. The position of the double bond is indicated by solid arrows above the MS trace. Migration of the proton from carbon atom 10 to 9 during double bond cleavage is shown by the dotted arrow.

by L8-14C (pVT100-U:Δ9-3) transformants when cultivated in unsupplemented medium would be <20% of total fatty acids, compared with values of 65–75% for the *OLE1* yeast strain or L8-14C (pVT100-U:*ole1/ole2*) transformants. This low level of unsaturated fatty acids would appear to have been insufficient to alleviate the yeast *ole1* defect. Low levels of 16:1(n-7) to 18:1(n-7) elongation were observed in all strains, especially when cultures were fed with 16:1(n-7), but the amount of 18:1(n-7) would have no significant effect on the growth studies described above. Both *OLE1* and *ole1* Δ9-3 transformants were also fed with a range of saturated and unsaturated fatty acids (18:3, γ-18:3, 20:0, 20:3, 20:4, 22:0 and 24:0) to test if the Δ9-3 protein could utilize other fatty acids as desaturation substrates, but in no case was bioconversion observed.

One major difference seen in yeast Δ9-3 transformants compared with control strains containing empty vector was the presence of hexacosenoic acid (26:1). Hexacosanoic acid (26:0) is normally found in *S. cerevisiae* at low concentrations (Welch & Burlingame, 1973; Oh *et al.*, 1997), mainly as the hydroxylated fatty acid moiety of sphingolipids (Kohlwein *et al.*, 2001). Although the amounts of 26:0 and 26:1 in yeast transformants were extremely low (<1% of total fatty acids), the appearance of 26:1 always correlated with the presence of the Δ9-3 gene (Table 2). 26:1 was also found in yeast *ole1* transformants containing the *M. alpina ole1* or *ole2*

genes (Table 2). The double bond in 26:1 was determined in all cases to be in the n-9 position by DMOX derivatization (Fig. 5). In the mass spectrum for 26:1, the molecular ion of 447 *m/z* units was clearly visible. Radical-induced cleavage occurred uniformly along the alkyl chain, releasing the methyl group at position n-1 (a loss of 15 *m/z* units) and methylene groups (-CH₂-) from positions n-2 onwards (losses of 14 *m/z* units). The n-9 position of the double bond in 26:1 was evident from the loss of 12 *m/z* units between positions n-10 and n-11 (320 and 308 *m/z* units, respectively). Further confirmation that the double bond was indeed at this position came from the characteristic increased abundance of ions resulting from cleavage between positions n-7 and n-8 (348 *m/z* units) and n-11 and n-12 (294 *m/z* units). This suggested that the three *M. alpina* Δ9-desaturases, *ole1p*, *ole2p* and Δ9-3, also displayed n-9 desaturase activity with very long-chain saturated fatty acid substrates. Conversely, fatty acid elongation of 18:1(n-9) to 26:1(n-9) in these yeast transformants could not be discounted, but this activity would have to be associated in some way with the overproduced Δ9-desaturase. High levels of an *M. alpina* Δ9-desaturase in yeast, as would be expected from the multicopy expression vector used in this study, may have stimulated the activity of endogenous fatty acid elongases (Elo2p/Elo3p), perhaps by interacting with the postulated fatty acid elongase complex in the ER membrane (Kohlwein *et al.*, 2001).

Table 3. Synthesis of very long-chain fatty acids in *ELO3* and *elo3* *S. cerevisiae* transformants

All cultures were grown at 25 °C for 4 days in unsupplemented YNB medium except for strain L8e1 (*ole1 elo3*) containing empty vector pVT100-U which was grown with added 16:1/18:1. Fatty acids were transmethyated as described above and concentrated samples were analysed by GC-MS. The relative amount of each fatty acid was calculated from the GC peak area and expressed as a percentage of total fatty acids. Sc, *S. cerevisiae*; Ma, *M. alpina*.

Yeast genotype	Δ^9 -desaturase gene in pVT100-U	Total fatty acids (%)							
		20:0	20:1	22:0	22:1	24:0	24:1	26:0	26:1
<i>OLE1 ELO3</i>	Empty	0.02	0.01	0.01	0	0.01	0	0.72	0
	Δ^9 -3	0.06	0	0	0	0	0	0.77	0.71
<i>ole1 ELO3</i>	Sc <i>OLE1</i>	0	0.25	0	0.02	0	0	0.30	0.06
<i>OLE1 elo3</i>	Empty	1.18	0.12	8.29	0.59	0.19	0.09	0	0
	Δ^9 -3	1.09	0.14	3.39	1.82	0	0.20	0	0
	Sc <i>OLE1</i>	0.15	0.59	1.41	0.14	0	0	0	0
<i>ole1 elo3</i>	Empty	1.22	0.11	6.67	0.60	0.21	0.13	0	0
	Sc <i>OLE1</i>	0.13	0.57	0.74	0.10	0	0	0	0
	Ma <i>ole1</i>	0.09	1.98	1.03	0.57	0	0	0	0

Fig. 6. Gas chromatograms of DMOX-derivatized fatty acids from *M. alpina* CBS 528.72 grown at 25 °C for 7 days in S2lowN medium (a) and in S2GYE medium (inset b). Peaks (i) and (ii) represent two isomers of 18:2 (Δ^9,Δ^{12} and Δ^5,Δ^9 , respectively). Peaks marked with an asterisk are unidentified. The expected retention times for 26:0 and 26:1 are indicated by arrows.

To determine if monounsaturations of long-chain fatty acids was simply a consequence of overexpressing any fatty acid Δ^9 -desaturase in this yeast system, the endogenous *S. cerevisiae* Ole1p was overproduced in both YN99-90 and L8-14C from vector pVT100-U. In addition, Δ^9 -3, *M. alpina* *ole1* and *S. cerevisiae* *OLE1* were overexpressed in *ELO3*-deleted strains, YD15281 (*OLE1 elo3*) and L8e1 (*ole1 elo3*), to examine the substrate specificity of desaturation/elongation reactions. Yeast *elo3* strains are blocked in 24:0 to 26:0

fatty acid elongation, but tend to accumulate higher levels of 20:0 and 22:0 compared with *ELO3* strains (Oh *et al.*, 1997; Kohlwein *et al.*, 2001; Fig. 4d). Overexpression of the *S. cerevisiae* *OLE1* gene in strain L8-14C resulted in a low but measurable amount of 26:1 which was relatively less than that found with the *M. alpina* genes (Table 3; see also supplementary figure at <http://mic.sgmjournals.org>). Similarly, the levels of 22:1 were higher in yeast *elo3* transformants containing *M. alpina* genes than in those containing the cloned *S.*

cerevisiae *OLE1* gene (Fig. 4e–f; Table 3), indicating that *M. alpina* $\Delta 9$ -desaturases have higher activity with longer chain fatty acid substrates. However, in all these transformants, fatty acid elongation from 18:0 (and probably 18:1) to 24:0 (and hence 24:1) occurred by the activity of the *ELO2* gene product. It was therefore not possible to distinguish whether the appearance of n-9 monounsaturated fatty acids in yeast transformants was a consequence of true n-9 desaturase activity or rather an effect of abnormally high levels of desaturase protein on fatty acid elongation.

The *in vivo* function of $\Delta 9$ -3 in *M. alpina* has yet to be determined. It is unlikely to be a pseudogene, derived from the duplication of *ole1* or *ole2*, since its primary transcript showed correct splicing of both introns to generate mature mRNA and its gene expression was highly regulated under different growth conditions (A. T. Carter, unpublished results). In particular, $\Delta 9$ -3 expression was induced significantly by increasing the ratio of carbon to nitrogen in the culture medium, a growth condition that leads to enhanced oil accumulation. Fatty acid profiles of *M. alpina* grown under high and low C:N conditions (C:N molar ratios of 857 and 34, respectively) showed very few differences, however, especially in the levels of monounsaturated fatty acids (Fig. 6). GC-MS analysis of DMOX-derivatized fatty acids revealed that *M. alpina* produced only small amounts of 24:0 with no detectable peaks for longer chained fatty acids such as 26:0 (Fig. 6a). The only significant difference between the two growth conditions was the relative increase in one of the two isomers of 18:2, viz. 18:2($\Delta 5, \Delta 9$), itself an unusual isomer, during growth in low C:N medium (Fig. 6b).

In conclusion, we suggest that $\Delta 9$ -3 encodes a third fatty acid $\Delta 9$ -desaturase that appears to have diverged from the other two *M. alpina* $\Delta 9$ -desaturases and now displays a different substrate specificity. Its function *in vivo* in *M. alpina* has yet to be established. The recent development of a transformation system for *M. alpina* (MacKenzie *et al.*, 2000) should facilitate the deletion of specific genes. This will help to determine which of these fatty acid desaturase genes are essential for cell viability and also elucidate their role in particular LCPUFA biosynthetic pathways.

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