Two fatty acid $\Delta 9$ -desaturase genes, *ole1* and *ole2*, from *Mortierella alpina* complement the yeast *ole1* mutation

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Genes encoding two distinct fatty acid $\Delta 9$ -desaturases were isolated from strains of the oleaginous fungus *Mortierella alpina*. Two genomic sequences, $\Delta 9$ -1 and $\Delta 9$ -2, each containing a single intron, were cloned from strain CBS 528.72 while one cDNA clone, LM9, was isolated from strain CBS 210.32. The $\Delta 9$ -1 gene encoded a protein of 445 aa which shared 99% identity with the LM9 gene product. These proteins also showed 40–60% identity to the $\Delta 9$ -desaturases (Ole1p) of other fungi and contained the three conserved histidine boxes, C-terminal cytochrome *b₅* fusion and transmembrane domains characteristic of endoplasmic reticulum membrane-bound $\Delta 9$ -desaturases. LM9 and $\Delta 9$ -1 are therefore considered to represent the same gene (*ole1*). The *ole1* gene was transcriptionally active in all *M. alpina* strains tested and its function was confirmed by complementation of the *Saccharomyces cerevisiae ole1* mutation. Fatty acid analysis of yeast transformants expressing the CBS 210.32 *ole1* gene showed an elevated level of oleic acid (18:1) compared to palmitoleic acid (16:1), the major fatty acid component of wild-type *S. cerevisiae*. This indicated that the *M. alpina* $\Delta 9$ -desaturase had a substrate preference for stearic acid (18:0) rather than palmitic acid (16:0). Genomic clone $\Delta 9$ -2 (*ole2*) also encoded a protein of 445 aa which had 86% identity to the $\Delta 9$ -1 and LM9 proteins and whose ORF also complemented the yeast *ole1* mutation. The transcript from this gene could only be detected in one of the six *M. alpina* strains tested, suggesting that its expression may be strain-specific or induced under certain physiological conditions.

Keywords: *Mortierella alpina*, $\Delta 9$ -desaturase genes, yeast complementation, fatty acid desaturation, oleaginous fungus

INTRODUCTION

The oleaginous zygomycete *Mortierella alpina* produces up to 50% of its cell dry weight as triacylglycerol oil, approximately 40% of which consists of the long-chain

polyunsaturated fatty acid (LCPUFA) arachidonic acid (ARA; 20:4, n-6). LCPUFAs are important both nutritionally and pharmacologically and there is much interest in developing microbial processes for their production (Ratledge, 1993; Sancholle & Lösel, 1995; Leman, 1997). They are directly incorporated into membranes of the central nervous system and hence affect brain and nerve development (Willatts *et al.*, 1998). LCPUFAs also act as precursors to a range of hormones, especially the prostaglandins, leukotrienes and thromboxanes, and are therefore thought to play important roles in combating or preventing a number of human diseases (Katayama & Lee, 1993). Oil produced by *M. alpina* has been screened for toxicity and is

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; 20:3, dihomogamma-linolenic acid; 20:4, arachidonic acid (ARA); ER, endoplasmic reticulum; LCPUFA, long-chain polyunsaturated fatty acid; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

The GenBank/EMBL accession numbers for the sequences reported in this paper are Y18553 and Y18554 (CBS 528.72 *ole1* and *ole2* genomic sequences, respectively) and AF0085500 (CBS 210.32 *ole1* cDNA).

currently used as a supplement in infant formulae in some countries (Hempenius *et al.*, 1997). The pathway for fatty acid desaturation and elongation from stearic acid (18:0) to LCPUFAs in filamentous fungi has been elucidated both by biochemical means and by studying mutant strains isolated by classical mutagenesis (Ratledge, 1993; Certik *et al.*, 1998).

Known fungal fatty acid desaturases are all endoplasmic reticulum (ER) membrane-bound enzymes which have their active site on the ER's cytoplasmic face. The active site comprises three histidine-rich boxes, normally containing eight essential histidine residues, which fold up to form the di-iron binding site in the native protein (Shanklin *et al.*, 1994). Cytochrome b_5 is used as the electron donor and in the majority of cases the desaturase is a protein fusion with a cytochrome b_5 domain attached at either the N or C terminus. The substrate for the initial Δ^9 -desaturation of 18:0 to oleic acid (18:1) is stearyl-CoA but some of the subsequent desaturation steps, including Δ^{12} - and Δ^6 -desaturation, occur using the fatty acyl chains of phospholipid molecules (Jackson *et al.*, 1998). *M. alpina* mutants defective in most of the desaturase activities have been isolated and these have been used, combined in some instances with specific desaturase inhibitors, to alter the fatty acid composition of the fungus (Jareonkitmongkol *et al.*, 1994; Kawashima *et al.*, 1997). An alternative approach to manipulating the LCPUFA biosynthetic pathway in *M. alpina* is to isolate and either overexpress or disrupt the genes encoding the desaturases and elongases. Indeed, the gene encoding the Δ^5 -desaturase which converts dihomono- γ -linolenic acid (20:3) to 20:4 has been isolated from two different strains of *M. alpina* (Knutzon *et al.*, 1998; Michaelson *et al.*, 1998). The *M. alpina* Δ^5 -desaturase, as predicted, contains three histidine boxes, although one of the essential histidine residues has been replaced with a glutamine, a change which is found in some other desaturases. This enzyme also contains a cytochrome b_5 domain fused at the N terminus.

The Δ^9 -desaturase carries out the first step in the desaturation pathway which leads to the greatest decrease in fatty acid transition temperature compared to subsequent desaturation reactions (Harwood, 1997). Because of this, Δ^9 -desaturase activity is important in maintaining membrane fluidity and its expression is therefore highly regulated. In several organisms, including *Saccharomyces cerevisiae*, this control is exerted both at the transcriptional and post-transcriptional level (Choi *et al.*, 1996; Gonzalez & Martin, 1996). The Δ^9 -desaturase gene (*OLE1*) has been isolated from a number of yeasts and filamentous fungi and all have a similar structure (Stukey *et al.*, 1990; Gargano *et al.*, 1995; Meesters & Eggink, 1996; Anamnart *et al.*, 1997; GenBank accession no. AF026401). We have therefore undertaken to isolate the gene encoding this enzyme from *M. alpina* and to study its expression. Recently, a Δ^9 -desaturase gene has been isolated from a patented strain of *M. alpina* whose gene product displays Δ^9 -desaturase activity in *Aspergillus oryzae* and

has a high degree of identity to other Δ^9 -desaturases (Sakuradani *et al.*, 1999). In this paper, we describe the isolation and characterization of two distinct Δ^9 -desaturase genes, *ole1* and *ole2*, from two strains of *M. alpina* which are freely available from fungal culture collections.

METHODS

Strains, media and growth conditions. *M. alpina* strain CBS 528.72 (ATCC 32222) was obtained from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, and strain CBS 210.32 was a gift from Professor S. Shimizu, Kyoto University, Japan. Other *M. alpina* strains used in this study were CBS 224.37, CBS 250.53 and CBS 527.72 (ATCC 32221), all obtained from the CBS, and strain CCF 2639 from the Culture Collection of Fungi, Charles University, Prague, Czech Republic, which was kindly supplied by Professor R. Herbert, University of Dundee. *S. cerevisiae* strain L8-14C (a *ole1* Δ ::*LEU2 leu2-3 leu2-112 ura3-52 his4*; Stukey *et al.*, 1989) was supplied by Professor M. Schweizer, Institute of Food Research, Norwich. *S. cerevisiae* strains NCYC 1383 (a *his3* Δ *1 leu2-3 leu2-112 trp1-289 ura3-52*), NCYC 1662 (α *arg⁻ met⁻*), AY925 (a *ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100*) and FY1679-3A (a *ura3-52*) were kindly supplied by Bruce Pearson, Institute of Food Research, Norwich. PCR-amplified DNA fragments were cloned in *Escherichia coli* strains XL-1 Blue MRF' (Stratagene), TOP10 (Invitrogen) or DH5 α (Promega). Genomic and cDNA libraries were constructed using *E. coli* strains XL-1 Blue MRF' (Stratagene) and ER1647 (Amersham Pharmacia Biotech), respectively. *In vivo* excision of phagemids was carried out using *E. coli* XL0LR (Stratagene) or BM25.8 (Amersham Pharmacia Biotech). *M. alpina* was maintained as vegetative mycelial cultures on potato dextrose agar slopes (PDA; Difco) and liquid cultures were inoculated with mycelial suspensions prepared in potato dextrose broth (PDB; Difco). PDB or GY broth, containing 2% (w/v) glucose, 0.5% (w/v) yeast extract, 0.05% (w/v) KCl, 2% (v/v) ACM salts (Morrice *et al.*, 1998), pH 6.5, were used for growing mycelium for nucleic acid extractions. The effects of fatty acid addition to *M. alpina* cultures were studied by supplementing PDB with 0.5% (v/v) ethanol with or without individual fatty acids at a final concentration of 1 mM. Cultures were grown either at 25 or 28 °C with shaking at 150 r.p.m. *S. cerevisiae* L8-14C was maintained on YPD agar supplemented with 0.5 mM palmitoleic acid, 0.5 mM oleic acid and 1% (w/v) tergitol NP-40 (Sigma) and transformants were selected on similarly supplemented yeast nitrogen base medium (YNB), containing 0.67% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose and 1.5% (w/v) agar, which lacked uracil.

Amplification of Δ^9 -desaturase probes and DNA sequencing.

Degenerate primers with homology to conserved histidine-box and cytochrome b_5 regions of Δ^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 combinations P3 (5'-TAYCAYAAAYTTY-CAYCA-3') and P4 (5'-TYSCCSCSGGRTGNTC-3'), and DESfor (5'-CTKGGYATYACWGCWGG-3') and DESrev (5'-CAGAASGTSGCRTCCTGGTG-3') were used to amplify fragments from 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 (primers P3/P4) or 46 °C (primers DESfor/DESrev) for 1.5 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. Degenerate primers His2for (5'-WSICAYMGAIYICAY-

CA-3') and His3rev (5'-YTCRTGRTGRAARTTTRTG-3') were used at an annealing temperature of 55 °C to amplify fragments from cDNA reverse-transcribed from total RNA of CBS 210.32 as described by Michaelson *et al.* (1998). Primers specific to *M. alpina* gene $\Delta 9$ -1, 91for (5'-CATCACAGCA-GGCAAGTAAC-3') and 91rev (5'-GGCGCCGAGCAGTG-CGAGCA-3'), were used at an annealing temperature of 62 °C to amplify a $\Delta 9$ -1-specific probe. Primer BR99 (5'-AAACG-TTATTACAACAGGC-3') was used in combination with primer 91for at an annealing temperature of 52 °C to generate the remaining part of the $\Delta 9$ -1 gene. PCR products were cloned into pCRII, pCR2.1-TOPO (both Stratagene) or pGEM-T (Promega) and the plasmids purified using the Plasmid Mini Purification kit (Qiagen). RT-PCR was carried out using a cDNA first-strand synthesis kit (Amersham Pharmacia Biotech) with an anchored oligo(dT)₁₈ primer, CN95 [5'-CTTCTGGATGTGCGTACTCGAGCT(T)₁₈-3'], followed by PCR with gene-specific primers. DNA sequencing was performed using the PRISM dye terminator kit (Perkin Elmer) and automated sequencer models 373A or 377 (Perkin Elmer). DNA sequences were analysed using the University of Wisconsin GCG software package.

Nucleic acid manipulations. High molecular mass genomic DNA was isolated from 4-d-old, PDB-grown *M. alpina* mycelium which had been freeze-ground with liquid nitrogen. DNA was extracted either by the Nucleon Phytopure Plant DNA Extraction kit (Amersham Pharmacia Biotech) or by a standard phenol/chloroform procedure (Michaelson *et al.*, 1998). In the latter, DNA was purified either by CsCl/ethidium bromide density gradient equilibrium centrifugation or by using a modification to the Plasmid Midi Purification kit (Qiagen) where the DNA, dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.0) was diluted approximately 10-fold in modified QBT buffer (Qiagen), lacking 2-propanol and Triton X-100, prior to application to the Qiagen column. Total RNA was isolated either by using the RNeasy Plant Mini kit (Qiagen) with freeze-ground mycelium or the TRIzol reagent (Life Technologies) with freeze-dried mycelium. Southern and Northern blotting were performed using standard procedures for either capillary or vacuum transfer of nucleic acids to nylon membranes. In all cases, hybridization was carried out at 65 °C in Puregene HYB-9 DNA hybridization solution (Flowgen) and blots were subsequently washed in 2 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 0.5% (w/v) SDS and in 0.1 × SSC, 0.5% (w/v) SDS at 65 °C. Signals were detected with a Fuji BAS 1500 phosphorimager or by autoradiography on X-ray film. On Northern blots, signals were standardized for fluctuations in RNA loading against the histone H4 transcript (P. Wongathanarat and others, unpublished results).

Library construction and screening. A genomic library was constructed in λ ZAP Express (Stratagene) with BamHI-digested DNA prepared from strain CBS 528.72 following the manufacturer's instructions. The library was screened with PCR-amplified $\Delta 9$ -desaturase probes which had been labelled with [α -³²P]dATP using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). Approximately 5 × 10⁴ p.f.u. was used in the primary screen and positive plaques subjected to a secondary screen before *in vivo* excision of the pBK-CMV phagemids with ExAssist helper phage in *E. coli* XL0LR (Stratagene). A cDNA library was constructed in λ MOSS/ox (Amersham Pharmacia Biotech) using EcoRI end-adapted cDNA synthesized from strain CBS 210.32 as described previously (Michaelson *et al.*, 1998). About 1.5 × 10⁵ p.f.u. from this library was screened with $\Delta 9$ -desaturase probes which had been labelled with [α -³²P]dCTP

and positive clones *in vivo* excised in *E. coli* BM25.8 (Amersham Pharmacia Biotech), a P1 *cre* recombinase host (Michaelson *et al.*, 1998). The 5' termini of positive cDNA clones were confirmed or their missing sequences completed by 5'-RACE (rapid amplification of cDNA ends) using the terminal transferase RACE method (Boehringer Mannheim) with cDNA as template and the nested primers RA15' (5'-AGAGTCGATGGTAACCTGCTGT-3') and RA25' (5'-GATACCAAGTCCCGTAGC-3'). The 3' termini of cDNA clones were completed by 3'-RACE using first-strand cDNA synthesized from 5 µg total RNA with the Ready-To-Go T-primed First-Strand kit (Amersham Pharmacia Biotech), according to the manufacturer's instructions. PCR was performed using a modified *ole1* primer RA13' (5'-GCGAATTCTCATCTACTGSCCTTTGTCA-3') which contains an *EcoRI* site (underlined) for cloning purposes and primer cDNA3en (5'-AACTGGAAGAATTCGCGGCCGC-AGGAAT-3') which is complementary to the *NotI* anchor region (underlined) at the 3' end of the first-strand cDNA.

Construction of yeast expression vectors. The LM9 and $\Delta 9$ -2 ORFs were cloned into the yeast expression vector pVT100-U which contains the 2µ origin of replication, the *URA3* selection marker and the alcohol dehydrogenase (*ADH1*) promoter and terminator regions (Vernet *et al.*, 1987). A synthetic, intronless version of $\Delta 9$ -2 with *Bam*HI and *Hind*III sites at the 5' end and a *Bam*HI site at the 3' end was created by overlap extension PCR using genomic clone $\Delta 9$ -2 as template and the following primer combinations: primer A (5'-AAGGATCCAAGCTTAAAAAATGGCCACTCCCCCTCCCCCA-3') and primer B (5'-CCATAACCGGTGGTATCCTGCAGTAATACCAAGGC-3'), and primer C (5'-GCCTTGGTATTACTGCAGGATACCACCGGTTATGG-3') and primer D (5'-AAGGATCCCTACTCTTCCTTGAATGGTCGCCATATA-3') where the start and stop codons and the overlap regions are single-underlined and the relevant restriction sites are double-underlined. The two PCR products, 310 and 1098 bp respectively, were then fused using primers A and D to generate a 1.3 kb intronless fragment. 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 was digested with *Hind*III and *Bam*HI and directionally cloned into pVT100-U. The sequence of the insert was checked using primers from the *ADH1* promoter and terminator regions: primer ADH1 (5'-GCTATCAAGTATAAATAGAC-3') and primer ADH2 (5'-GAAATTCGCTTATTTAGAAG-3'), respectively.

Since LM9 is a cDNA clone, PCR was carried out with this as template to create a *Hind*III site at the 5' end of the ORF and an *Xba*I site at the 3' end using primer E (5'-AATCTAGAAAGCTTAAAAAATGGCAACTCCTCTTCCCCCTC-3') and primer F (5'-AATCTAGACTATT-CGCGCTTGACGTGGTCAGTGCC-3') at an annealing temperature of 58 °C. The 1366 bp PCR fragment was digested with *Hind*III and *Xba*I, directionally cloned into pVT100-U and the sequence of the insert checked using primers ADH1 and ADH2.

Yeast transformation and fatty acid analysis. pVT100-U containing either the LM9 or $\Delta 9$ -2 ORF was transformed into the *S. cerevisiae ole1* mutant strain L8-14C by the lithium acetate/single-stranded carrier DNA/PEG whole cell method of Gietz *et al.* (1995). Undigested vector DNA (200 ng) in sterile TE buffer (pH 8.0) and 250 µg single-stranded herring sperm carrier DNA were incubated with 50 µl competent yeast cells for each transformation. Following lithium acetate/PEG treatment and heat shock at 42 °C for 15 min, the cells were

resuspended in 1 ml YNB broth and aliquots plated onto fatty-acid-supplemented YNB agar to select for the *URA3⁺* marker. *URA3⁺* colonies were restreaked onto non-supplemented YNB agar to check for complementation of the *ole1* mutation.

Two independent LM9 and $\Delta 9$ -2 yeast transformants were grown in 10 ml YNB broth at 25 °C for 4 d for fatty acid analysis. Untransformed L8-14C and transformants containing pVT100-U without an insert were grown in fatty-acid-supplemented YNB broth with and without 20 mg uracil l⁻¹. $\Delta 9$ -2 transformants were also grown in YNB broth supplemented individually with 0.5 mM linoleic acid (18:2), α -linolenic acid (18:3), γ -linolenic acid (γ -18:3), dihomogamma-linolenic acid (20:3) or arachidonic acid (20:4) to test for desaturation of other substrates. In all cases, cells were washed three times with distilled water at 45 °C and freeze-dried prior to transmethylation of the fatty acids. Methanolic HCl (2 ml; Supelco) was added to each dried sample and refluxed at 80 °C for 1 h. Fatty acid methyl esters were extracted with 1.5 ml 0.9% (w/v) NaCl/1 ml hexane and finally resuspended in 0.5 ml hexane. GC analysis was carried out on a BPX70 0.25 μ m column (SGE) in a Perkin Elmer AutoSystem GC, with a column temperature of 50–250 °C and an injection port temperature of 250 °C. The helium carrier gas pressure was 141 kPa.

RESULTS AND DISCUSSION

Isolation of $\Delta 9$ -desaturase genes from *M. alpina* strains CBS 528.72 and CBS 210.32

Degenerate primer combination DESfor/DESrev, covering a region containing histidine boxes 1 and 2, generated several fragments with CBS 528.72 genomic DNA as template, two of which, 594 and 554 bp in size, showed 40–60% amino acid identity when translated to known fungal $\Delta 9$ -desaturases. Both fragments contained consensus sequences for introns (described below) of 155 and 115 bp, respectively, which disrupted the ORF at the same position in the deduced proteins. These protein fragments showed about 85% identity to each other, suggesting that there could be two $\Delta 9$ -desaturase genes in this strain of *M. alpina*. A smaller fragment of 232 bp, generated with specific primers 91for and 91rev, which were derived from the 594 bp fragment sequence, still contained the 155 bp intron and was subsequently used to probe a genomic library from CBS 528.72. Degenerate primer combination P3/P4, which was designed to amplify a region further downstream between histidine box 3 and the haem-binding consensus sequence of the cytochrome *b₅* domain, generated a fragment of 347 bp that also had sequence homology to $\Delta 9$ -desaturase genes. This fragment was also used to screen the CBS 528.72 genomic library. Similarly, degenerate primer combination His2for/His3rev, annealing to histidine boxes 2 and 3 respectively, amplified a 426 bp fragment from CBS 210.32 cDNA which on translation had 40–60% amino acid identity to fungal $\Delta 9$ -desaturases and this was used to probe a cDNA library from CBS 210.32.

Clone LM9 was isolated from the CBS 210.32 cDNA library and contained a 1.66 kb *Eco*RI insert which encoded a protein of 445 aa with 98% identity to the $\Delta 9$ -

Fig. 1. Structural organization of $\Delta 9$ -desaturase genes from *M. alpina*. Comparison of (a) *ole1* from CBS 210.32 (cDNA clone LM9) with (b) *ole1* (genomic clone $\Delta 9$ -1) and (c) *ole2* (genomic clone $\Delta 9$ -2) both from CBS 528.72 (sizes are not to scale). Each ORF is indicated by one or two open boxes with the size of the encoded protein (aa) within each box. Percentage values refer to the amino acid identity of the given protein with the CBS 210.32 $\Delta 9$ -desaturase. The three conserved histidine boxes are marked 1, 2 and 3, respectively: 1, HRLWAH; 2, HRAHH; 3, HNFHH. The haem-binding pocket of the cytochrome *b₅* domain, EHPGG(X)₁₀DMT(X)₉HS, is indicated by *b₅*. The approximate position and size (nt) of the single introns in CBS 528.72 *ole1* and *ole2* are also shown. The lengths of the putative 5'- and 3'-UTRs of the *ole1* genes are given (nt). B, *Bam*HI. The arrows indicate the direction and approximate positions of the primers used in this study: a, DESfor; b, DESrev; c, 91for; d, 91rev; e, BR99; f, P3; g, P4; h, His2for; i, His3rev; j, RA15'; k, RA25'; l, RA13'; m, cDNA3en.

desaturase from *M. alpina* 1S-4 described by Sakuradani *et al.* (1999) and 40–60% identity to other fungal $\Delta 9$ -desaturases. This protein displayed the three conserved histidine boxes, C-terminal cytochrome *b₅* fusion and transmembrane domains characteristic of ER membrane-bound $\Delta 9$ -desaturases (Fig. 1a). The poly(A) tail and part of the 3'-untranslated region (3'-UTR) were missing from this clone. Several attempts at 5'-RACE using gene-specific nested primers RA15' and RA25' only extended the 5' end of the cDNA by 32 bp, suggesting that the transcription start site was close to this point. 3'-RACE with primers RA13' and cDNA3en completed the 3'-UTR and included the poly(A) tail. A consensus poly(A) addition signal, AATAAA (Gurr *et al.*, 1987), was present in this gene 160 bp downstream from the TAG stop codon. On assembling the complete LM9 sequence, the total length of the cDNA was 1.74 kb. On Southern blots of CBS 210.32 genomic DNA, digested with either *Bam*HI or *Hind*III and probed with fragment His2for/His3rev, at least two strongly hybridizing bands were seen per track, indicating that this strain may also have more than one $\Delta 9$ -desaturase gene (data not shown).

The 232 bp 91for/91rev and 347 bp P3/P4 genomic probes did not cross-react with each other under stringent hybridization conditions but hybridized to

Table 1. Fatty acid composition of *ole1* and *ole2* transformants of *S. cerevisiae* L8-14C

Cultures were grown at 25 °C for 4 d in unsupplemented YNB medium. Fatty acids were transmethylated directly from washed, freeze-dried cells and fractionated by GC. The values given are the relative fatty acid compositions as a percentage of the total fatty acid content as determined by GC peak area. Values for the wild-type *OLE1*⁺ strains represent the mean of four determinations while those for the *ole1* and *ole2* transformants represent the mean from two independent yeast transformants (± SD).

Yeast strain	Fatty acid composition (%)				
	16:0	16:1	18:0	18:1	18:1/16:1 ratio
Wild-type <i>OLE1</i> ⁺ strains*	18.6 ± 2.7	39.5 ± 4.1	5.9 ± 1.2	21.6 ± 4.2	0.6 ± 0.2
L8-14C + pVT100-U: CBS 210.32 <i>ole1</i>	19.9 ± 0.9	5.8 ± 0.8	2.5 ± 1.1	53.6 ± 0.5	9.4 ± 2.0
L8-14C + pVT100-U: CBS 528.72 <i>ole2</i>	32.0 ± 1.6	3.4 ± 0.8	4.8 ± 0.4	43.8 ± 4.0	13.9 ± 6.3

* Mean values from four haploid, wild-type *OLE1*⁺ strains (NCYC 1383, NCYC 1662, AY925 and FY1679-3A) grown under identical conditions.

*Bam*HI fragments of approximately 2.9 and 3.4 kb, respectively, on Southern blots of CBS 528.72 genomic DNA (data not shown). On probing the genomic library with each fragment, positive clones were purified, insert sizes confirmed by *Bam*HI digestion and the inserts sequenced. Clone Δ9-1, isolated using the 91for/91rev probe, contained a 2903 bp *Bam*HI fragment whose encoded protein shared 99% amino acid identity with the LM9 protein. This clone did not contain the complete ORF since the *Bam*HI site is located about 260 bp upstream of the stop codon in LM9. The remaining part of the Δ9-1 gene was isolated by PCR using primers specific to the Δ9-1 intron (primer 91for) and the LM9 3'-UTR (primer BR99) with CBS 528.72 genomic DNA as template. The DNA sequence of this PCR fragment was 100% identical to that of the genomic Δ9-1 clone up to the *Bam*HI site and 99% identical to the LM9 sequence downstream of this site. Clones Δ9-1 and LM9 were therefore considered to represent the same gene, designated *ole1*. The 155 bp intron in Δ9-1 has the relatively rare 5' splice site GCAAGT also found in the *M. alpina* 1S-4 Δ9-desaturase gene (Sakuradani *et al.*, 1999), ends with CAG at the 3' splice site and contains the consensus lariat sequence TGCTAAC 42 nt from the 3' end (Gurr *et al.*, 1987). This intron disrupts the ORF in a highly conserved region of Δ9-desaturases and its removal was confirmed *in vivo* by RT-PCR analysis and sequencing using primers DESfor and DESrev which flank the intron. The structural organization of the CBS 528.72 *ole1* gene is illustrated in Fig. 1(b).

Genomic clone Δ9-2 was isolated using P3/P4 as probe and contained a 3342 bp *Bam*HI insert which also encoded a protein of 445 aa. This protein showed less identity (86%) to the *M. alpina* *ole1* gene product. The single intron of 115 bp disrupted the ORF in the same position as in Δ9-1 but had the more common 5' splice site GTATGT, a 3' splice site, TAG, and consensus lariat

sequences CATCAAC and TCTCAAC, 42 and 18 bp, respectively, from the 3' end (Gurr *et al.*, 1987). A less common poly(A) addition signal, AT(A)₆TAATAA, was located 23 bp downstream from the TAG stop codon. The organization of this gene, designated *ole2*, is outlined in Fig. 1(c).

Functional characterization of the *M. alpina* Δ9-desaturase genes in *S. cerevisiae*

To confirm the *in vivo* function of the two putative Δ9-desaturase genes, the CBS 210.32 *ole1* and CBS 528.72 *ole2* ORFs were expressed in L8-14C, an *ole1* mutant of *S. cerevisiae* (Stukey *et al.*, 1989). Each ORF was cloned into the yeast expression vector pVT100-U (Vernet *et al.*, 1987) with the consensus sequence (A)₆ immediately 5' of the ATG start codon of each gene, a sequence which is associated with highly expressed *S. cerevisiae* genes (Hamilton *et al.*, 1987). Both ORFs formed transcripts in yeast transformants, as determined by Northern analysis (data not shown), and complemented the *ole1* mutation since the transformants grew without 16:1 and 18:1 supplementation. Fatty acid analysis of the transformants showed that both 16:1 and 18:1 were produced but that the ratio of 18:1 to 16:1 was higher than in wild-type *S. cerevisiae* (Table 1). *M. alpina* only produces negligible amounts of 16:1 and this result confirms that the *M. alpina* Δ9-desaturases had a substrate preference for 18:0 compared with 16:0, unlike the *S. cerevisiae* enzyme. There also appeared to be a difference in fatty acid composition between the *ole1* (LM9) and *ole2* (Δ9-2) transformants, indicating that the 14% difference in amino acid identity between the two proteins may have some significance for Δ9-desaturase activity. *ole2* (Δ9-2) yeast transformants fed with a range of unsaturated fatty acids failed to desaturate these further, confirming that the *ole2* protein only had Δ9-desaturase activity (data not shown).

Fig. 2. Expression of (a) *ole1* and (b) *ole2* in six strains of *M. alpina*. Total RNA (20 µg), extracted from 4-d-old PDB cultures grown at 25 °C, was Northern-blotted. The membranes were first probed with either (a) the 232 bp *ole1* fragment 91for/91rev or (b) the 347 bp *ole2* fragment P3/P4, both from CBS 528.72, and exposed for 72 h on a phosphorimage plate. The same membranes were then probed with a 208 bp fragment from the *M. alpina* histone H4.1 ORF and the phosphorimage plate exposed for 5 h to determine RNA loading.

Δ9-desaturase gene expression in *M. alpina*

Transcription of the *ole1* and *ole2* genes was examined in a number of *M. alpina* strains by Northern analysis. In all six strains studied, the *ole1* transcript was detected but showed strain to strain variation in amount relative to the histone H4 loading control (Fig. 2a). The two strains from which the *ole1* gene had been isolated, CBS 210.32 and CBS 528.72, both produced significant amounts of *ole1* mRNA. On the other hand, expression of the *ole2* gene could only be detected in strain CBS 527.72 (Fig. 2b). The cDNA version of this gene was absent from the CBS 210.32 cDNA library and the failure to detect *ole2* transcript by RT-PCR with RNA extracted from strains CBS 210.32 or CBS 528.72 confirmed that *ole2* was inactive or extremely poorly expressed in these two strains of *M. alpina*. Sequence comparisons of the CBS 528.72 *ole1* and *ole2* promoter regions and 5'-UTRs showed that there was no hom-

Fig. 3. Effect of fatty acid supplementation on *ole1* expression. Transcript levels of *ole1* were measured in 4-d-old PDB cultures of CBS 210.32 grown at 28 °C which had been supplemented 3 h prior to harvesting with 0.5% (v/v) ethanol (Control) or 0.5% (v/v) ethanol plus one of a number of fatty acids at a final concentration of 1 mM. Total RNA (10 µg) was Northern-blotted and probed with the 426 bp *ole1* fragment His2for/His3rev from CBS 210.32. Signals were detected by autoradiography on X-ray film. RNA loading was determined by probing with the histone H4.1 fragment.

ology between the *ole1* and *ole2* 5' regions and this is most likely the basis of this differential gene expression. Preliminary PCR analysis of genomic DNA from CBS 210.32 and a third strain of *M. alpina*, CBS 527.72, which was the only strain to show *ole2* gene expression (Fig. 2b), using *ole2*-specific primers revealed that both strains possessed an *ole2* gene. The 528 bp fragment which was amplified had 97% DNA sequence identity with the corresponding region of the CBS 528.72 *ole2* gene and contained a 118 nt intron at the same position (data not shown). This suggests that most, if not all, strains of *M. alpina* contain both *ole1* and *ole2* genes.

The presence of two distinct Δ9-desaturase genes in one species is not unique. Two linked genes encoding Δ9-desaturases, which show differential expression, have been identified in *Drosophila* (Wicker-Thomas *et al.*, 1997), mouse (Tabor *et al.*, 1998) and *Arabidopsis* (Fukuchi-Mizutani *et al.*, 1998). Sesame and rose also possess two Δ9-desaturase genes which are differentially expressed under specific growth conditions but their linkage has not yet been determined (Fukuchi-Mizutani *et al.*, 1995; Yukawa *et al.*, 1996). Humans, on the other hand, have two Δ9-desaturase genes but one is an inactive, intronless pseudogene which contains several mutations (Zhang *et al.*, 1998). The need for two distinct Δ9-desaturases in *M. alpina* is not known but in other systems it appears to be related to differentiation and gene expression in specific organs or tissues (Wicker-Thomas *et al.*, 1997; Fukuchi-Mizutani *et al.*, 1998).

Supplementation of CBS 210.32 cultures with a variety of unsaturated fatty acids containing a Δ9-unsaturated bond reduced *ole1* transcript levels, with 18:1, 18:2 and γ-18:3 having the most pronounced effect (Fig. 3). This repression has been observed in several fungi (Choi *et al.*, 1996; Meesters & Eggink, 1996). In *S. cerevisiae* the

regulatory sequences responsible for transcriptional control, the 111 bp FAR (fatty-acid-regulated) element containing repeated CCCGGG motifs and the sequence GGGGTTAGC, have been identified in the *ole1* promoter (Choi *et al.*, 1996). In addition, an as yet undefined sequence in the 5'-UTR is required for post-transcriptional regulation of the *S. cerevisiae ole1* gene (Gonzalez & Martin, 1996). Similar sequences could not be found in the promoter regions or 5'-UTRs of the *M. alpina ole1* and *ole2* genes.

Conclusions

(1) Two distinct $\Delta 9$ -desaturase genes, *ole1* and *ole2*, have been isolated from the oleaginous fungus *M. alpina*. The *ole1* gene product showed 98% amino acid identity to that of the *M. alpina* $\Delta 9$ -desaturase gene described by Sakuradani *et al.* (1999) and 40–60% amino acid identity to other fungal $\Delta 9$ -desaturases. The *ole2* gene product had lower identity (86%) to the *ole1* gene product.

(2) Both *ole1* and *ole2* ORFs complemented the *ole1* mutation in *S. cerevisiae* and showed a substrate preference for 18:0 compared with 16:0.

(3) The *ole1* gene was expressed in all strains of *M. alpina* which were studied and showed transcriptional regulation in response to supplementation with $\Delta 9$ -unsaturated fatty acids.

(4) Transcription of the *ole2* gene was only detected in one of the six strains of *M. alpina* which were examined, suggesting that gene expression may be strain-specific or induced under certain physiological conditions.

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