Malic enzyme: the controlling activity for lipid production? Overexpression of malic enzyme in *Mucor circinelloides* leads to a 2.5-fold increase in lipid accumulation

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Malic enzyme (ME; NADP⁺-dependent; EC 1.1.1.40) has been postulated to be the rate-limiting step for fatty acid biosynthesis in oleaginous fungi in which the extent of lipid accumulation is below the maximum possible. The genes encoding the isoform of ME involved in fatty acid synthesis were identified in *Mucor circinelloides* and *Mortierella alpina*, two commercially useful oil-producing fungi, using degenerate primers. Both showed high similarity with ME genes from other micro-organisms. The whole-length ME gene from each source was cloned into a leucine auxotroph of *Mc. circinelloides* and placed under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase gene (*gpd1*) promoter. After confirming correct expression of the ME genes, the two recombinant strains were grown in fully controlled, submerged-culture bioreactors using a high C:N ratio medium for lipid accumulation. Activities of ME were increased by two- to threefold and the lipid contents of the cells, in both recombinants, were increased from 12% of the biomass to 30%. Simultaneously, the degree of fatty acid desaturation increased slightly. Thus, increased expression of the ME gene leads to both increased biosynthesis of fatty acids and formation of unsaturated fatty acids, including γ -linolenic acid (18:3 n-6). At the end of lipid accumulation (96 h), ME activity in the recombinant strains had ceased, as it had done in the parent wild-type cells, indicating that additional, but unknown, controls over its activity must be in place to account for this loss of activity: this may be due to the presence of a specific ME-cleaving enzyme. The hypothesis that the rate-limiting step of fatty acid biosynthesis is therefore the supply of NADPH, as generated specifically and solely by ME, is therefore considerably strengthened by these results.

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INTRODUCTION

Accumulation of lipid in organisms is of considerable current interest: in animals, excess lipid accumulation leads to obesity; in plants, higher seed-oil production would be advantageous; and in micro-organisms, lipid production is now commercially achieved for oils rich in arachidonic acid (ARA; 20:4 n-6) and in docosahexaenoic acid (DHA;

22:6 n-3) (Cohen & Ratledge, 2005). The latter two products are currently used in infant formula preparations and the demand for them is now exceeding the present production capacity (Ratledge, 2005; Ratledge & Hopkins, 2006a, b). Although the process of fatty acid biosynthesis is now well known, the genetic factors that control the extent to which lipid accumulates in a particular organism are far from clear (almost invariably the lipid that is accumulated by animals, plants and micro-organisms is in the form of triacylglycerols, the chief components of which are the fatty acyl chains).

In both plants and micro-organisms, the extent of lipid accumulation is determined by the genetic constitution, as maximum attainable lipid contents can vary enormously between species and even between individual strains. In micro-organisms, the range of lipid contents amongst the so-called oleaginous species can vary from 20–25% to >70% (Ratledge, 1997). The oil content of commercial plant seeds can range from ~15%, as with soybeans, to

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Abbreviations: ACL, ATP, citrate lyase; GLA, γ -linolenic acid; ME, malic enzyme.

The GenBank/EMBL/DDBJ accession numbers for the ME sequences of *Mc. circinelloides* and *Mt. alpina* are DQ975377 and DQ973624, respectively.

A table showing the strains and plasmids used in this study is available with the online version of this paper.

~50 % with groundnuts (peanuts) and other oil-seed crops. Various attempts have been made with plants to increase the flux of carbon into fatty acid biosynthesis and to identify possible bottlenecks, with subsequent genetic modification being carried out to remove postulated impediments to lipid accumulation. None of these attempts has been successful, with only relatively small increases (~10–15 %) in lipid accumulation being recorded (see for example Broun *et al.*, 1999; Thelen & Ohlrogge, 2002; Rangasamy & Ratledge, 2000; Ramli *et al.*, 2005) following genetic manipulation.

For some time it has seemed to us that the overall regulation of fatty acid biosynthesis must lie outside the flux of carbon in view of this lack of success in over-expressing various supposed rate-limiting enzymes of fatty acid synthesis (Wynn & Ratledge, 1997; Wynn *et al.*, 1999; Ratledge & Wynn, 2002; Ratledge, 2004). We have previously described the process of lipid accumulation in both oleaginous yeasts and filamentous moulds as requiring the key activity of ATP : citrate lyase (ACL) in order for cells to produce a sufficient supply of acetyl-CoA in the cytosol (Botham & Ratledge, 1979; Evans & Ratledge, 1985). Although this activity is essential for lipid accumulation to take place (i.e. lipid to exceed more than 20 % of the cells), it is insufficient to explain the range of lipid levels in the oleaginous cells, as there is no correlation between ACL activity and lipid accumulation (Ratledge & Wynn, 2002). This cytosolic enzyme is, however, absent in non-oleaginous yeasts such as *Saccharomyces cerevisiae* and *Candida utilis* (Botham & Ratledge, 1979). Overexpression of the gene encoding ACL in tobacco plants fails to increase lipid content by more than 15 % of that of the control plants (Rangasamy & Ratledge, 2000). (It should be noted that at that time it was not possible to carry out similar genetic modifications of oleaginous micro-organisms, as none of these species had been established as genetically manipulatable).

Of key significance, at least in our view, has been the role of malic enzyme (ME; EC 1.1.1.40) in lipid accumulation. This enzyme carries out the irreversible decarboxylation of malate to pyruvate with the formation of NADPH from NADP⁺. It is then this NADPH which is vital for fatty acid biosynthesis. No other NADPH-generating enzyme appears able to provide the requisite reducing power for fatty acid synthase to function (Wynn & Ratledge, 1997; Wynn *et al.*, 1999). We have shown using *Mucor circinelloides* as a model organism that sesamol, as a specific inhibitor of ME activity, decreases lipid accumulation from 25 % of the cell biomass to 2 % without adversely affecting growth (Wynn *et al.*, 1997). Moreover, there is a direct correlation between the decreasing activity of ME during the lipid-accumulation phase and the extent of lipid accumulation in both this fungus and the related *Mortierella alpina* (Wynn *et al.*, 1999): when ME activity ceases, lipid accumulation stops. Thus, an explanation of why different organisms have different levels of their maximum lipid contents can be provided by the expression and possible control of ME.

Further, a mutant of the fungus *Aspergillus nidulans* that lacks ME activity has only 12 % lipid when grown under exactly the same conditions as the wild-type which has ME activity and accumulates 30 % of its biomass as lipid (Wynn & Ratledge, 1997). Although we have also shown that whilst there are several isoforms of ME present in *Mc. circinelloides* (Song *et al.*, 2001), only one of these (termed isoform III) is involved in NADPH formation and is linked to the lipid-accumulation process. This isoform III is converted into isoform IV, presumably post-transcriptionally, upon the commencement of lipid accumulation, which is triggered by depletion of nitrogen from the growth medium. We have isolated and sequenced another isoform (type II) of ME from *Mc. circinelloides* (Li *et al.*, 2005), but this is the form of ME associated with anaerobic metabolism and not with fatty acid biosynthesis.

In this present paper, we have now identified the gene encoding ME isoforms III/IV and, having isolated and sequenced it, have reintroduced it into *Mc. circinelloides* under the control of a constitutive promoter. In this way we have been able to achieve expression of a higher activity of ME in the fungus, with the result that the content of lipid in the cells has increased from 12 % of the biomass to 30 %. However, ME activity still disappears by the end of the lipid-accumulation phase, with the result that lipid accumulation, though more than doubled, does not continue indefinitely. A possible reason for this is discussed later in the paper.

METHODS

Strains, media and growth conditions. *Mc. circinelloides* CBS 108.16 and *Mt. alpina* Peyron CBS 696.70 were used for ME gene cloning and analysis. The leucine auxotrophic strain *Mc. circinelloides* R7B (kindly provided by Dr Santiago R. Torres Martines, University of Murcia), a derivative of strain *Mc. circinelloides* f. *lusitanicus* CBS 277.49 (syn. *Mucor racemosus* ATCC 1216b) (Schipper, 1976), was used as the recipient in transformation experiments and as a control strain for gene expression with 20 µg leucine ml⁻¹ in its medium. *Escherichia coli* strain InVzF' (Invitrogen) was used for DNA manipulation. *E. coli* strain LE392 (Promega) was used for genomic library construction. A list of the strains and plasmids used in this work is given in Supplementary Table S1.

Mt. alpina and *Mc. circinelloides* were maintained on solidified Kendrick medium (Kendrick & Ratledge, 1992) at 30 °C and stored at 4 °C. *Mc. circinelloides* R7B was grown at 30 °C on YPG complete medium (3 g yeast extract, 10 g peptone and 20–50 g glucose per litre distilled water) or, for plate cultures, in the minimal medium YNB (0.5 g Difco yeast nitrogen base without amino acids, but with 1.5 g ammonium sulphate l⁻¹, 1.5 g glutamic acid l⁻¹ and 10 g glucose l⁻¹) with the glucose added post-sterilization. Fungi (about 0.3 × 10⁵ spores of each strain) were initially cultivated in 1 l stirred bottles containing 800 ml Kendrick medium for 16 h at 30 °C, and then inoculated at 10 % (v/v) into a 5 l fermenter containing 4 l Kendrick medium modified to contain 2 g ammonium tartrate l⁻¹ and 50 g glucose l⁻¹. Leucine at 20 µg ml⁻¹ was included in the medium used for *Mc. circinelloides* R7B as control. Fermenters were held at 30 °C, stirred at 500 r.p.m. with aeration at 0.5 volume of air per volume of fermenter per minute (v/v min⁻¹), and pH was maintained at 5.5–6.5 by automatic addition of 2 M KOH or 2 M HCl.

Nucleic acid manipulation. High-molecular-mass genomic DNA was extracted from mycelia of *Mt. alpina* CBS 696.70 and *Mc. circinelloides* CBS 108.16, and grown for 24 h in Kendrick medium which had been freeze-ground with liquid N₂. A standard phenol/chloroform extraction procedure (Michaelson *et al.*, 1998) was used prior to application of a DNA Clean-up kit (Promega), and the final DNA was dissolved in Tris/EDTA buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.0). Total RNA was isolated by using the RNeasy Plant Mini kit (Qiagen) with freeze-ground mycelium following the manufacturer's instructions. Southern and Northern blotting were performed using standard procedures for capillary transfer of nucleic acids to nylon membranes. DNA was labelled with [α -³²P]dCTP or a fluorescent labelling kit (Amersham). In all experiments, hybridization was carried out at 55 °C in a hybridization buffer, and the blots were subsequently washed at 55 °C successively with 2 × saline sodium citrate (SSC), 1 × SSC and 0.5 × SSC containing 0.5 % SDS (SSC=0.15 M NaCl, 15 mM sodium citrate, pH 7.0). Signals were detected by autoradiography on X-ray film. Northern blot signals were corrected for fluctuations in RNA loading by comparison with band densities obtained from concurrent electrophoresis in an ethidium bromide-stained formaldehyde-containing agarose gel.

Amplification of fragments of the ME gene from *Mt. alpina* and *Mc. circinelloides*. Degenerate primers were designed according to the homology with conserved amino acid sequences of ME and were obtained commercially (MWG-Biotech) as sense primers P1 [5'-GT(AGCT)GT(AGCT)AC(AGCT)GA(CT)GG(AGCT)CA(AG)-3'] and P2 [5'-GG(AGCT)AT(ACT)CC(AGCT)GTTGG(AGCT)AAA-3'], and antisense primers P3 [5'-(AG)TT(AGCT)GC(AG)AA(AG)T-C(CT)TC(AG)AA(CT)TG-3'] and P4 [5'-(AGCT)CC(CT)TG(AGT)-AT(AG)TC(AG)TC(AG)TT(AG)AA-3']. Two DNA fragments, 310 and 370 bp, were amplified using *Mt. alpina* and *Mc. circinelloides* genomic DNA, respectively, by primers P2 and P4. PCR conditions were 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 44 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were cloned into the pGEM-T Easy vector (Promega) and the resulting plasmids (named pFMt6 and pFMc4.1 for *Mt. alpina* and *Mc. circinelloides*, respectively) were purified using a Wizard column (Promega). A list of all plasmids used in this work is given in Supplementary Table S1.

Genomic library construction and screening. Two genomic libraries were constructed using LambdaGME-11 Genomic Cloning Vector (*Bam*HI arms) (Promega) with *Sau*3AI-digested genomic DNA prepared from *Mc. circinelloides* CBS 108.16 and *Mt. alpina* CBS 696.70, following the manufacturer's instructions. The libraries were screened with Amersham Redivue [α -³²P]dCTP-labelled PCR-amplified probe from *Eco*RI-digested pFMt6 and pFMc4.1. Approximately 2 × 10⁵ p.f.u. was used in each primary screen. Eleven positive plaques were obtained in the secondary screen from the *Mt. alpina* genomic library and 13 from that of *Mc. circinelloides*.

Transformation conditions. Transformation of *E. coli* strain InV α F' (Invitrogen) was used in this study. Competent cells for transformation were obtained according to the manufacturer's instructions and transformed by heat shock at 42 °C for 90 s. The transformants were selected on LB plates with 50 µg ampicillin ml⁻¹ and 160 µg X-Gal ml⁻¹. Plasmids were isolated by growing the cells in liquid LB medium and using a Wizard column (Promega).

Transformation of *Mc. circinelloides* R7B was carried out as described elsewhere (van Heeswijk & Roncero, 1984) with the modifications of Wolff *et al.* (2002), as follows. Protoplasts were prepared by enzymic treatment of germlings with a mixture of 125 µg chitosanase-RD (US Biologicals) and 5 U chitinase (from *Streptomyces griseus*, Sigma) in a total volume of 2 ml. Cell wall digestion was carried out for 2–3 h at

28 °C. Typically, 2–5 µg DNA was used per transformation. Transformants were selected on YNB plates maintained at room temperature for 2 days.

DNA sequencing and bioinformatic analysis. The DNA sequences from both strands were determined with the Sequencing kit (Beckman Coulter) and a capillary electrophoresis sequencer (CEQ 8000, Beckman Coulter) according to the manufacturer's instructions. The sequence data were interpreted and aligned by CodonCode Aligner. The position of introns was speculated by bioinformatic analysis using MacVector software (Oxford Molecular), and the sequences were confirmed by cDNA sequences.

Construction of expression vectors. The episomal *E. coli*–*Mc. circinelloides* shuttle vector pEUKA11, containing the *E. coli kan*^R gene under the transcriptional control of the *Mc. circinelloides* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd1*) promoter and terminator, was a kind gift from Dr Jose Arnau at the Bioteknologisk Institut, Hoersholm, Denmark (see Appel *et al.*, 2004; Wolff *et al.*, 2002).

Each full-length *malEMt* and *malEMc* gene (from *Mt. alpina* and *Mc. circinelloides*) was cloned separately into the expression vector pEUKA11 to replace the *kan*^R gene under the control of the *gpd1* promoter and terminator. Overexpression of *malEMt* and *malEMc* was achieved by a high level of constitutive gene expression in *Mc. circinelloides* R7B under the control of the *gpd1* promoter and terminator (see Fig. 1).

To construct overexpression vectors, a synthetic version of each *malEMt* and *malEMc* with a *Xho*I site at the 5' end and a *Not*I site at the 3' end was created by overlap-extension PCR using pMtX1.4 or pMcP3.7 (which are, respectively, the corresponding subclones of one of the positive lambda clones of *Mt. alpina* and *Mc. circinelloides*) as template and the primers MtTailedF (5'-GCATTGTATTCTCGAGATGGCTCTCTCCAGTCTCTCC-3'), MtTailedR (5'-TGTCTACATGGCGGCCGCGAAAACTTAGAGGTGAGG-3'), McTailedF (5'-AATCATTCCTCTCTCGAGCTCTCAACAGAAATGTGCG-3') and McTailedR (5'-CTATATTGCGGCCGCTACTACAACACTACAATTACCAGC-3'), where the relevant restriction sites are underlined. All extension PCRs were carried out as follows: 5 min at 94 °C, 30 cycles of 94 °C for 0.5 min, 55 °C for 1 min and 72 °C for 3 min, and a final 10 min extension at 72 °C.

The 3 kb *Mt. alpina* and 2.1 kb *Mc. circinelloides* ME gene fragments were created and inserted into the pGEM-T Easy vector (Promega). The resulting plasmids (pMtME and pMcME) were digested with *Xho*I and *Not*I, and the fragments containing the full-length *malEMt* and *malEMc* genes were then inserted into the linear pEUKA11 vector digested by *Xho*I and *Not*I and gel-purified to remove the *kan* gene. In the resulting plasmids (pEUKMt2.6 and pEUKMc4.8), the individual ME gene was placed under the control of the promoter and terminator regions of the *gpd1* gene; the nucleotide sequences of the fusion plasmids were confirmed by sequencing.

Isolation of single *Mc. circinelloides* transformants with pEUKMt2.6 and pEUKMc4.8. Transformed primary colonies were isolated on YNB plates (pH 3) to select for leucine-independent growth. Well-isolated colonies were transferred from the original transformation plates to fresh YNB plates and, after appropriate growth of the successive vegetative cycles, sporangiospores were harvested.

Determination of ME activity. Biomass was harvested by filtration under reduced pressure through a Whatman no. 1 filter, washed with distilled water and then suspended in extraction buffer [100 mM KH₂PO₄/KOH, pH 7.5, containing 20 % (w/v) glycerol, 1 mM benzamidine and 1 mM DTT]. After being disrupted by a single pass

Fig. 1. Construction of overexpression vectors. The extensive full-length *malEMt* gene (a) and *malEMc* gene (b), with a *XhoI* site at the 5' end and a *NotI* site at the 3' end, were inserted into the expression vector pEUKA11 (c) (a gift from Dr Jose Arnau, Biotechnologisk Institut, Hoersholm, Denmark) to replace the *kan* gene under the control of *gpd1* promoter and terminator, yielding the plasmids pEUKMt2.6 and pEUKMc4.8 (not shown). The grey boxes in the regions encoding *malEMt* and *malEMc* denote introns; P, promoter; T, transcription terminator region; *leuA*, *Mc. circinnelloides* leucine marker; *Ap^R*, ampicillin-resistance gene; *gpd1*, glyceraldehyde-3-phosphate dehydrogenase gene.

through a One-Shot cell disrupter (Constant Systems) at 64 MPa, the material was centrifuged (10 000 *g* for 10 min at 4 °C) and the supernatant used immediately for the determination of ME activity (see Wynn *et al.*, 1997). Protein was determined using the Bradford method with BSA as a standard.

Determination of cell dry weight, and glucose and ammonium ion concentrations. Biomass was harvested by filtration through a pre-weighed glass-fibre filter (Whatman GF/A), washed twice with distilled water and dried at 110 °C to a constant weight. Glucose in the culture medium was determined using a glucose oxidase Perid test kit (Boehringer Mannheim) and ammonium using the indophenol method (see Song *et al.*, 2001).

Analysis of cell lipid and fatty acids. Lipid was extracted from freeze-dried biomass as previously described (Song *et al.*, 2001) and its amount determined gravimetrically. Fatty acids, as their methyl esters, were prepared and analysed by GC, as described by Wynn & Ratledge (2000).

Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma.

RESULTS

Isolation of ME genes (*malE*) from *Mt. alpina* and *Mc. circinnelloides* CBS 108.16

To clone the ME genes from *Mt. alpina* and *Mc. circinnelloides*, four degenerate primers (P1, P2, P3 and P4) were kindly designed by Dr J. P. Wynn according to the conserved amino acid sequences identified after multiple alignment of 14 known ME sequences from a wide variety of sources (including mammals, micro-organisms and plants) at the National Centre for Biotechnology Information (NCBI) database. These four degenerate primers were used for PCR amplification of genomic DNA of *Mt. alpina* and *Mc. circinnelloides*, and the resulting 310 and 370 bp DNA fragments were cloned into the pGEM-T Easy vector (Promega) and sequenced. The deduced amino acid sequences from these DNA fragments showed high similarities to those of MEs from various organisms on BLAST analysis (data not shown).

These two amplified DNA fragments were separately used as probes to isolate corresponding lambda clones from *Mt. alpina* and *Mc. circinnelloides* CBS 108.16 genomic libraries. Screening of the genomic libraries resulted in the isolation of 13 positive clones for *Mt. alpina* from 6×10^4 plaques and 11 positive clones for *Mc. circinnelloides* from 2×10^5 plaques. One of the clones from the *Mt. alpina* genomic library had a 22 kb DNA fragment containing the *Mt. alpina* ME gene and one from the *Mc. circinnelloides* genomic library had a 23 kb DNA fragment containing the *Mc. circinnelloides* ME gene. A 4.5 kb DNA fragment from the former and a 5.5 kb DNA fragment from the latter were obtained from each positive lambda clone after digestion by *XhoI* or *PstI*, respectively, and were inserted into *XhoI*- or *PstI*-digested plasmid pBluescript SKII (pBSK) (Stratagene). The resulting plasmids, pMtX1.4 and pMcP3.7, were purified, each inserted sequence was then determined, and genes were named *malEMt* and *malEMc*, respectively. The ATG codons in the *Mt. alpina* DNA fragment at nt 1006 and in the *Mc. circinnelloides* DNA fragment at nt 2373 were assumed to be the translation initiation codons for each gene. This conclusion was based on their positions relative to the promoter elements, notably CCAAT-, TATA- and CT-rich regions, and the consensus sequence generated by using these frames. Therefore, the consensus sequences of the putative ME genes from *Mt. alpina* and *Mc. circinnelloides* were considered to be, respectively, 2983 and 2104 bp.

Two introns (436 and 537 bp) in *malEMt* and four introns (69, 57, 56 and 59 bp) in *malEMc* were confirmed by cDNA sequencing using RT-PCR products as templates. The putative promoter regions (CCAAT-, TATA- and CT-rich regions) existed within 200 bp upstream of the translation initiation site (ATG) of both *malEMt* and *malEMc*.

Genomic Southern blot analysis

Southern blots of *Mt. alpina* CBS 696.70 genomic DNA, digested with *XhoI*, *EcoRI* and *BamHI*, and *Mc. circinnelloides*

CBS 108.16 genomic DNA, digested with *Pst*I, *Eco*RI and *Bam*HI, and hybridized with the respective full-length gene, were performed. Only one strong signal was obtained from each hybridization after film development, indicating that both fungal strains have a single gene copy (data not shown).

Analysis of *malEMt* and *malEMc* sequence

GenBank database searches with the deduced amino acid sequences of MEs from *Mt. alpina* and *Mc. circinelloides* revealed close matches to the complete amino acid sequences of MEs from a number of yeast and fungi. In an alignment, the putative amino acid sequences of the *Mt. alpina* ME showed, respectively, 49, 50, 47, 45, and 45 % identity to those from *Neurospora crassa* (Galagan *et al.*, 2003), *A. nidulans* (Galagan *et al.*, 2005), *Gibberella zeae* PH-1 (B. Birren and others, unpublished work; see http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html), *Magnaporthe grisea* (Dean *et al.*, 2005) and *Dictyostelium discoideum* (Eichinger *et al.*, 2005). In a similar alignment, the putative amino acid sequence of the *Mc. circinelloides* MEs showed, respectively, 42, 44, 43, 42 and 45 % identity to the same five organisms.

The alignment showed that there was 55 % identity between the amino acid sequences of *Mt. alpina* and *Mc. circinelloides*, and that the identical residues were scattered throughout the sequence.

From these alignments, regions of functional significance could be found (Fig. 2). Consensus sequences in the primary structures of the two MEs revealed that two highly conserved dinucleotide binding sites, GXGXXG/A (highlighted in Fig. 2), are present regardless of the cofactor specificities of the enzyme (Wierenga *et al.*, 1986; Rothermel & Nelson, 1989; Borsch & Westhoff, 1990). Regions Y(138) to C(146) in *Mc. circinelloides* and Y(194) to C(202) in *Mt. alpina* (highlighted in Fig. 2) matched perfectly with the binding site for malate [numbered Y(44)–C(52)] given by Kulkarni *et al.* (1993). The sequences from D(199) to D(231) in *Mc. circinelloides* and from D(256) to D(288) in *Mt. alpina* (highlighted in Fig. 2) could be assigned to the consensus sequence for the site at which the ADP ring of NADP⁺ is bound, which has a predicted $\beta\alpha\beta$ secondary structure [numbered D(193)–D(223) in Wierenga *et al.*, 1986]. Furthermore, the crystal structures of the NAD(P)⁺-dependent ME from human mitochondria (Yang *et al.*, 2000) and an NADP⁺-dependent ME from pigeon cytosol (Yang *et al.*, 2002) (neither shown in Fig. 2) have been reported; various residues, F, E, D (numbered as 347, 348 and 349, respectively, in the *Mt. alpina* sequence) and F, N, D and D (369–372 in the same sequence and highlighted in Fig. 2) are also highly conserved and are probably involved in the binding of a divalent metal ion. The K(220) residue in *Mc. circinelloides* and the K(277) residue in *Mt. alpina* (numbered Lys183 in the pigeon ME by Yang *et al.*, 2002) may be important residues for catalytic activity. In addition, the 2'-phosphate group of NADP⁺ would be predicted to interact with S(387) in *Mc.*

circinelloides and S(440) in *Mt. alpina* (numbered Ser346 by Yang *et al.*, 2002). Many of these residues are highly conserved in MEs from a wide range of species. For example, E(291), D(292) and D(315) in *Mc. circinelloides* correspond to K(456) in *Mt. alpina* (numbered Lys362 in the pigeon enzyme in Yang *et al.*, 2002), and these residues are always highly conserved in NADP⁺-dependent MEs (Yang *et al.*, 2002; Chang & Tong, 2003).

Construction of overexpression vectors of *malEMt* and *malEMc*

Episomal expression vectors, termed pEUKMt2.6 and pEUKMc4.8, were constructed by inserting the whole gene region of *malEMt* and *malEMc* to replace the *kan* gene under the control of the *gpd1* promoter and terminator of the vector pEUKA11 (see Methods). The structural maps of the *Mt. alpina* and *Mc. circinelloides* gene expression vectors are shown in Fig. 1.

Characterization of the transformed strains

(a) Identification of transformed *Mucor* strains by Southern blotting. To investigate the role that ME plays in lipid accumulation and fatty acid desaturation in *Mt. alpina* and *Mc. circinelloides*, the respective ME gene overexpression vectors, pEUKMt2.6 and pEUKMc4.8, were transformed into *Mc. circinelloides* R7B, which is a leucine auxotroph (see Methods). Seventeen transformants with pEUKMt2.6, named *Mucor-malEMt*, and nine transformants with pEUKMc4.8, named *Mucor-malEMc*, were obtained and analysed for being stable Leu⁺ phenotypes by successive cycles of vegetative growth in the absence of selective pressure. One transformant of each type was finally selected for Southern analysis using a LeuA gene fragment (*Pst*I-digested from plasmid pLEU4, kindly provided by Dr Santiago R. Torres Martines, University of Murcia) as a probe. The hybridization with genomic DNA from both mutant strains showed strong signals, while no signal was detected from genomic DNA of the wild-type strain (see Fig. 3). This result demonstrated that both overexpression vectors had been separately transformed into *Mc. circinelloides* R7B and that they carried both the leucine gene and the *malEMt* or *malEMc* gene.

The wild-type *Mc. circinelloides* R7B strain and the two transformants, *McmalEMt* and *McmalEMc*, were each grown in 5 l fermenters with nitrogen-limited medium (see Methods) for 96 h to engender lipid accumulation. For growth of the wild-type R7B strain (leucine auxotroph), 20 mg leucine l⁻¹ was also added. Transcription of the ME genes, ME activity, lipid content, fatty acid profile, cell dry weight, and ammonium and glucose concentration were determined throughout growth.

(b) Northern blot analysis. To examine the transcript levels of *malE* genes, total RNA was isolated from all three strains grown in the 5 l fermenters. Northern blotting was

<i>Mortierella</i>	MALSMSTLPRSSAARSFFLQKGNNSCLHSSSFCCYSTKISIPRHTLKPRLTNSLQSHTS	60
<i>Mucor</i>	-----MSPIIDFVRRQLSSTKLHEEQQTAT	25
<i>Gibberella</i>	-----MAALDQHRGY	10
<i>Magnaporthe</i>	-----MNQAAPTATIDIAQIEGIKMANLDTLRGY	30
<i>Aspergillus</i>	-----MRWIPSPGGSMLVSRGLLHKIRPSPSAQLPLSVFSSSFVFGSRCHSGA	49
<i>Dictyostelium</i>	-----	
<i>Neurospora</i>	-----MSHSQNPQDEQAQKGS	16
<i>Mitochondrial</i>	-----MLSLKRIATTTKSNIFHRALLAQR	24
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<i>Mortierella</i>	LAPLFSTRSTPSTPTTTHSIFRSLSSASVAIPDHLNTHQHTDNMTGTATPTELRSAL	120
<i>Mucor</i>	TNDLVS-----SGYLNEGKYEVRLNINAGCLQKKNLYIGTAMDPAKRQRL	72
<i>Gibberella</i>	VQDHYQE-----VKQERSPEYVDENNAAGYQIVREPLWNKGLAFTPEQRANK	57
<i>Magnaporthe</i>	SNTTPAP-----VQQAITEYTFMSQAAGYQVIREPMWNKGLMFTPEERVSK	77
<i>Aspergillus</i>	AVTQKSR-----MARFSPQSRPVHATPHVNHNGPIEQNYDTANPAYIRKYL	96
<i>Dictyostelium</i>	-----MQNKPSFILRNPSAN-----	15
<i>Neurospora</i>	KFSL-----PLSTSGPLECALKGTVLLNHPYFNKGSAFTKEERRDF	58
<i>Mitochondrial</i>	SYSYINA-----NEYS DPRHDKNVHVMHSGVNLHDP LLSKGTAFSIAERERL	73
	: :	
<i>Mortierella</i>	HLQGLTPAKVESFELQKKRALAQLRSKSSDIEKYVFLAWLRNTNVLRYGLVGDQLEVRE	180
<i>Mucor</i>	GLNGLLPAGVETLEIQKARALRVLRSHNLLKEYILMAQLRTTNVRLFYKIVIDELET--	130
<i>Gibberella</i>	NLTGLIPHTIESFDTQCARAMKMIQTRQTPIDKYLYSLTLKDQNTDLFYRLLDNVR--	115
<i>Magnaporthe</i>	NLTGLLPHTMETEDIACARAMKMIRSRSTNVDRYLYLSSLKSSNTDLFYRLLDNVR--	135
<i>Aspergillus</i>	RTYGLTPRPESYETQKTRCLAQLALKQTPIDKFLYSLTLRKNVHLFYRLVTDHLRE--	154
<i>Dictyostelium</i>	--KGLLPKVESLQEQSDRLSQFTSFNTNLERYIFLNCILDRNETLFYLLSNLLE--	71
<i>Neurospora</i>	ALHGLLPPIQSLQEQVQRAYEQYCSQPNDLAKNTEMTSMKEQNEVLFYRLLDHLE--	116
<i>Mitochondrial</i>	SIRGLVPPRCQEMDKQLLRINKRLDACETPLAKVFVLAALHDRNETLYYKIIMEHLE--	131
	** * : : * . : : : : * * : : :	
<i>Mortierella</i>	LDALNMTETLPLIYFTVGTACQNYSSIIYFPLAPPGQPDGLFLSINDLPNLTIQIIQNYKP	240
<i>Mucor</i>	-----VQLAPVIYFTVGTACLEYSTIYFPLAAGVPDGLYLTKAELPELQTTIRNYRP	184
<i>Gibberella</i>	-----LMPLVYFTPTIGDVCLQYSSIYT-----RPEALYISIKQKRSIRAMLRNWPC	161
<i>Magnaporthe</i>	-----LMPLVYFTPTIGDVCLQYSSLFT-----RPEALYISIKQKRSIKTMLANWFPY	181
<i>Aspergillus</i>	-----LTPLIYFTPVGEACQWSEIYQ-----QPEGMYLSWEDRGNLASVIANWFO	200
<i>Dictyostelium</i>	-----MMPIIYFTPVGEACQKFGNEFR-----FAQGMYFASQDKGNIRAMDNWFA	117
<i>Neurospora</i>	-----MFSVVYFTPTGEAIQNYSLRFR-----RPEGVFLNINDMSVKRDLAQWKG	162
<i>Mitochondrial</i>	-----LAGIIYFTVGLASQMSHSIYR-----RSRGMYFSSQDRGQMSAMVYNWF-	176
	: :	
<i>Mortierella</i>	FPQDPSLTPQIAVITDGSRIILGLGDLGVGMGIPVGKLQLYVAGAGIDPRRTLPIITLDLG	300
<i>Mucor</i>	TDTEG-FEPEIAVISDGSRIILGLGDLGTNGMGI PMGKLQLYVAGAGIDPRRTLPIITLDLG	243
<i>Gibberella</i>	Q-----DPEICVITDGSRIILGLGDLGINGVGISIGKLALYTGAAGIHPSKTLPIVLDCG	215
<i>Magnaporthe</i>	P-----NPEICVITDGSRIILGLGDLGINGVGISIGKLALYTAAAGIHPSKTLPIVLDCG	235
<i>Aspergillus</i>	P-----NVEITCITDGSRIILGLGDLGINGMGIPIGKLALYTTCAGIRPEATLPIITLDLG	254
<i>Dictyostelium</i>	EG-----VDIIVSDGSRIILGLGDLGTNGMGI PMGKLQLYVAGAGFCPTRTLPIITDGS	171
<i>Neurospora</i>	PE-----DIDYIVVTDGEIILGIDQCGGGLISIAKLVLMTICGGIHFNRLPVLVDCG	217
<i>Mitochondrial</i>	HD-----KVDVIVVTDGSRIILGLGDLGANGMEIPIGKLALYTVAAGIRPRAVLPIVVLVDCG	231
	: *	
<i>Mortierella</i>	TNNEKLKDEFYLGRLREKRAGDDKFFPFVDAVIEALTSYPKLLIQFEDFSSEHAFQLLD	360
<i>Mucor</i>	TNNEKLKDEFYILGRQKRPNDEEFYQTVDTVLTALHTVYPNLLIQFEDFSSEHAFGLLE	303
<i>Gibberella</i>	TNNEENLQDPFYLGRLQKRPSYPEQQA FMEDEFMEATREVPNMVVFEDFSEKAFGLD	275
<i>Magnaporthe</i>	TNNEENLRDPLYLGLRSKRPSVQEQQDFCEVMHSIKEYVPNMVVFEDFTEKAFNYLD	295
<i>Aspergillus</i>	TSNKALREDPLYLGSRRDKITPDEEREFLDELMAALTERWPGIVIQFEDFKN--PFPAL	312
<i>Dictyostelium</i>	TNTKKYLEDKYLLGERHPRI PDSEYYPLVDEFLAAAFNKWPKVIVQFEDISNDHCFNLLD	231
<i>Neurospora</i>	TNNEELLKDDLYLGLRQKRAGDKYDDFVETFIKAARELYPRAYIHFEFGLDNARRLE	277
<i>Mitochondrial</i>	TNNQDLLNDPLYLGMSPRLGEGEYYSFIDEWVTAITSRWPDTLIQFEDFKYPHAYNLLA	291
	* . : : . * * : : : : : : : : : : : : : : : *	
<i>Mortierella</i>	KYQPKKFC---FNDDIQGTGAVILAGFMNAINLAG---IPAKDHKILFFGAGSAAVCVA	413
<i>Mucor</i>	KYQNMQLC---FNDDIQGTGAVILSGVINAIKVEKENQVSPRDHRIVFYGAGSAAIGVA	360
<i>Gibberella</i>	RYRDQYRC---FNDDIQGTGAVVLGGYI GAVEQSN---VPIEEQRLVFMGAGSAGVGVA	328
<i>Magnaporthe</i>	RYRFDPKLNCMFNDDIQGTGAVVLAGYINAVKLSN---VPVEEQRLVFMGAGSAGAGVA	351
<i>Aspergillus</i>	RYQDVYTC---FNDDIQGTGAVILGGVINAVKRSN---VPCKDQRAVFFGAGSAGVGVA	365
<i>Dictyostelium</i>	EYRNKYLC---FNDDIQGTGSVILSGFINAVRSVQ---KPIKEHRMFLGAGSAGIGVA	284
<i>Neurospora</i>	RYRPMQPC---FNDDVQGTGCVTLAAIMAGLH---ISKQKLSVDRMVFGAGTAGVGIA	330
<i>Mitochondrial</i>	KYRNRTIC---FNDDIQSTSSITLAGILASLSRSG-MSQEDLSNERIICVAGSAGVGVC	347
	.* : *	

performed on cells taken after 24, 48 and 72 h using the ME gene-specific probes from pMt6 or pMc4.1 (see Fig. 4). The transcript level of the wild-type strain decreased rapidly after nitrogen exhaustion from the medium (before 24 h; see Fig. 5), but in the two transformants it remained high throughout growth and the subsequent lipid-

accumulation phase. Thus, in the wild-type strain, there is a rapid cessation of ME expression, thereby leading to loss of ME activity and consequent low lipid accumulation (see below). In the transformants, continued expression of ME was achieved by placing the gene under the control of the *gpd1* promoter.

<i>Mortierella</i>	KQLAAYFVNDEGMTE-DEAREMVVLVDSKGLVTLDRGD--KLAEHKLYFARKDNAGSQYP	470
<i>Mucor</i>	RQIQSYFQIEHNMT-EEAKHVFWIVDSKGLVTTTRGD--KLAQHKKVYARGDNEGQQYK	417
<i>Gibberella</i>	KQLVEYYT-RRGLSE-AAAREKFWLVDTKGLVTKDRGD--RLAEHKKYFARTDNNQQQFR	384
<i>Magnaporthe</i>	RQLVDYYRNAHGMSE-QEAKDKFYLVDTKGLITSDRFDGAKLP SHKRYFARDNNNGHQFQ	410
<i>Aspergillus</i>	KQIVAFFM-REGMTE-DEARSCFWLVDTKGLVTADRGD--KLAEHKLYFARTDNNAGQQFK	421
<i>Dictyostelium</i>	DCIMSLFD-EAGVSK-EEARKSFWFVDSKGLITTTTRGD--ELTSQKKQYAREDYT-YQLK	339
<i>Neurospora</i>	DHVRDAIAADADISP-EEAGKQIWLIDKPEGLTTKSESLSDAQKK---YAKSHDDWAN--	384
Mitochondrial	EGIDCMVAQGRVKSREBAYSKIYMLDQYGLLGNPGVHLP SDEEHGHPYQFRTTKLLDDRQ	407
	: : . * . : : * * : : : :	
<i>Mortierella</i>	S-----IASVIEHVRPTALFGLSSQSGAFSEDLKSMATLNQR-----PIV	511
<i>Mucor</i>	E-----LIDIVNYN-LYSLIGLSSTTGAFNTQVLERLASLNEQ-----PIV	457
<i>Gibberella</i>	T-----LEEVIYVKSALVGLTATFGVTFEPVVRALKSSVQEGGLE-RRPIL	431
<i>Magnaporthe</i>	T-----LEDVIEFARPSALVGLTATFGVTFESIVRGLKASVDAAGHRNRRPIL	458
<i>Aspergillus</i>	T-----LEEVDHVKPSILMGLSTQGGVFTPEILRKMAEWNTN-----PMI	462
<i>Dictyostelium</i>	S-----LLEVVRDVKPTAIIGLSGIGGSFSQVEIEEMAKHVEK-----PIV	380
<i>Neurospora</i>	-----EKTDL LAVI KEVPHILVGTSTKPKSFTEEVVRENAKHVER-----PII	428
Mitochondrial	YCYVKHDLRDQMGLEELVRKVKPTVLLGLTGIQGVXSEEAVERMAKHHER-----PVI	460
	: : : . * : : : : : * : :	
<i>Mortierella</i>	FPLSNPAYQAECSEFQAMIHKTGKVI FASGTAFPKYTDNPNTGL----ISAPGQGNMYIF	567
<i>Mucor</i>	FPLSNPATQAECTFEQAMEATNNKVI FASGTAFPAYTIKSTGE----VNTPGQGNMYIF	513
<i>Gibberella</i>	FPLSNPLTKAECTFEQAI EWTEGTVLFASGSPFPNPKAKPGDETHHTVYHFNQGNVYIF	491
<i>Magnaporthe</i>	FPLSNPLTKAECTFSQALDWTGTVI FASGSPFAPHTVKNGDS--ATYYPNQGNVYIF	516
<i>Aspergillus</i>	FPLSNPSSKSECD FETAVTNTDGRVLFASGSPFPTMFTNSAG-ETHMYYPGQGNMYIF	521
<i>Dictyostelium</i>	FALSNPTTNAECTAEQAYQWTDGRCI FASGSPFKPVEYKGTTF-----VPGQGNMYIF	434
<i>Neurospora</i>	LPLSNPTKLHEAVPEDLLNWDGKALVATGSPFDPVKGPWGKDGKEIEIDVAECNNSVVF	488
Mitochondrial	FPLSNPDTHAECTAEAEAFKWTGDAI FASGSPFKDVELPNGKIGR-----TNQCNNYSYSF	515
	: . * * * . * . . * . : . * : * : *	
<i>Mortierella</i>	PGLGLG--GILAQPETISDRLIYSAARACANALTVEERSQGLLYPVLPRIRQVSAEVA	625
<i>Mucor</i>	PGLGLG--ACLANPAHF-DRMIYEASKALADSLTEEBISKGWLYPSLN-YRVSVAIVAA	569
<i>Gibberella</i>	PGLGLG--AILAKASRVTDENVYTSAAALAGSLNADEVHKGGLIYPRIERVRDASVIVARE	549
<i>Magnaporthe</i>	PGIGLG--AILAKASRVTDENVYTSAAALAGSLNADEVHKGGLIYPRIERVRDASVIVARE	574
<i>Aspergillus</i>	PGIGLG--TILSKAVKVTDSMIYASGEALSKALTAEEIERGLLYPDLTRIKKVSVVARN	579
<i>Dictyostelium</i>	PGLGLA--ASVCEAKHVTDAII TAAKTLASFVEDSEVLTKGIYPGLQHIREISTRIAVK	492
<i>Neurospora</i>	PGIGLG--SVLSRANRVTDKMLMAASKGVAEMGPALRDDTAPLLPGVDVVRDVSVRVARR	546
Mitochondrial	PVSGLGXGIVTSRASRVTPNMFLETARTIADMATPSQLKEGILFPGVSQLEVALNVGR	575
	* ** . : . . : . : : : : : : : : : :	
<i>Mortierella</i>	VVTEAVAEGTATNEEAIK-LVKSHDKAALLNFVNSNMWTDGVQHEISTTFAPHL-----	679
<i>Mucor</i>	VQOETLNENLATSQAMMT-QCKSHED--ILDYVSAHMMSPD-YGNNSNSQAGKL-----	620
<i>Gibberella</i>	VMKAARRGGVSELPESQWAEWEEWGDVALTSFIKQHIYNPLCFADAKL-----	597
<i>Magnaporthe</i>	VMKCCRRQGF SKLDDLELWKSWEWGDNALMAWIKTKLYKPLKE-----	617
<i>Aspergillus</i>	VIRAAQEAQVDRD-----TLRTMDDETLDAWIKSRMYDPHTEVQSLEREVGSI LSTLGA	634
<i>Dictyostelium</i>	VIEKAYEEGMAQLP-----RPDNIEALVKSRQYVPSYDKSKN-----	529
<i>Neurospora</i>	VILAAMEEGVVEEKD-----VPTNEEDLDEWIREQMMDPVYRPLKYVEGNKASRGAKGD	600
Mitochondrial	VCEVAFEEGYATAK-----LEEGEILSEVVRSSAYQPEYVPLVTRDH-----	617
	* : : : .	
<i>Mortierella</i>	-----	
<i>Mucor</i>	-----	
<i>Gibberella</i>	-----	
<i>Magnaporthe</i>	-----	
<i>Aspergillus</i>	PQQFVNGSSDEAKL	648
<i>Dictyostelium</i>	-----	
<i>Neurospora</i>	LRVLGNPNDE---	611
Mitochondrial	-----	

Fig. 2. Multiple amino acid sequence alignments of ME from different eukaryotic micro-organisms. *Mortierella* and *Mucor* indicate the strains used in this work (see text); *Gibberella*=*G. zae* (accession no. EAA77789); *Magnaporthe*=*M. grisea* (accession no. XP 362590); *Aspergillus*=*A. nidulans* (accession no. EAA57954); *Dictyostelium*=*D. discoideum* (accession no. XP645111); *Neurospora*=*N. crassa* (accession no. XP960731); Mitochondrial=mitochondrial enzyme from *Mc. circinelloides* (accession no. AR209191; see also Li *et al.*, 2005). Symbols given below the sequences: *, identical amino acid residue; :, conserved amino acid; ., semi-conserved amino acid. The areas highlighted are: red, malate binding site; yellow, dinucleotide binding site; green, divalent metal ion binding site; other areas are described in the text.

(c) ME activities. ME activities in both the transgenic strains, together with that in the wild-type, are shown in Fig. 5. Activities of ME within cells at different stages of culture were consistently higher in the two transformants than in the original parent. Up to threefold higher activity was seen in the *McmalEMt* transformant and up to twofold in the *McmalEMc* transformant compared with the control (R7B) strain.

The overexpressed MEs were also detected by activity staining after non-denaturing PAGE (see Fig. 6). Unlike *Mc. circinelloides* CBS 108.16, which produces six ME isoforms (Song *et al.*, 2001), only two isoforms were observed in the *Mc. circinelloides* R7B strain, and these corresponded to isoforms III and IV of the former strain. These are the two isoforms associated with lipid accumulation (see Introduction). The activity of the smaller isoform

Fig. 3. Southern blot of *Pst*I-digested genomic DNA from *Mucor* strains. Lanes contained genomic DNA from (left to right): *Mc. circinelloides* R7B (control) (Wild), *Mucor-malEMc*, and *Mucor-malEMt*, which was hybridized with a ^{32} P-labelled *leuA*-specific probe. The arrow indicates the molecular mass of the signals, but the size of the bands was non-quantitative due to problems with DNA separation in the wells.

in each mutant strain (see Fig. 6) was stronger and remained longer than that in the control strain, and was thus instrumental in the increased accumulation of lipid in the transformants (see below).

(d) Lipid accumulation and fatty acid profiles. The pattern of growth, including exhaustion of NH_4^+ from the medium, was similar in the two transformants and in the control strain. NH_4^+ was depleted in all three cases before 24 h, and lipid accumulation probably commenced 2–4 h prior to this. Glucose consumption rates of the two transformants were faster than that of control strain after 48 h, and concomitantly the cell dry weights also increased more rapidly in the control strain (see Fig. 5). Significantly, both transformed strains accumulated considerably more lipid than the original wild-type strain (see Fig. 5), and this explains the increased uptake of glucose and the higher

biomass levels. The amounts of lipid accumulated in the transgenic strains *malEMt* and *malEMc* were, respectively, 2.5- and 2.4-fold more than in the control strain.

Not only was a much higher amount of lipid synthesized by the two transformants than by the original strain but also, again significantly, the higher activity of ME in the transformants maintained and even increased the amounts of unsaturated fatty acids produced in the lipid (see Table 1). Thus, increased ME activity led to increases of both lipid biosynthesis and unsaturated fatty acid production, including that of γ -linoleic acid (18:3 n-6), which is a feature of this mould.

DISCUSSION

ME (NADP $^+$ -dependent) is widely distributed in a variety of organisms and possesses a highly conserved primary structure, suggesting its physiological importance (Chang & Tong, 2003). To isolate the ME genes from *Mt. alpina* and *Mc. circinelloides*, degenerate primers were designed based on the amino acid sequence motifs conserved in multiple species, and the subsequent PCR product was used as probe to isolate MEs from each of the respective genomic libraries. From both cloned ME genes, *malEMt* and *malEMc*, CCAAT-, TATA- and CT-rich boxes, which are generally present in the promoter regions of eukaryotes, were identified within 200 bp upstream of the transcriptional initiation site (ATG). Two introns of 436 and 537 bp are present in *malEMt* from *Mt. alpina*, for which relatively large introns (up to 1 kb) have been reported (Wongwathanarat *et al.*, 1999; Mackenzie *et al.*, 2000). Four introns of 69, 57, 56 and 59 bp are present in *malEMc* from *Mc. circinelloides*, and for this species the mean size of an intron is 69 bp (Velayos *et al.*, 2000). The sequence of *malEMt* has a G + C content of 54 %, whereas the introns have a G + C content of 49 %. The sequence of *malEMc* has a G + C content of 56 %, with the introns having G + C contents of 36, 42, 41 and 39 %. These data fall within the

Fig. 4. Northern blot analysis of transcription of ME from *Mucor* strains. (a) *Mc. circinelloides* R7B (control; wild-type); (b) *Mucor-malEMt* (the transformed strain with the ME gene from *Mt. alpina* CBS 696.70); (c) *Mucor-malEMc* (the transformed strain with the ME gene from *Mc. circinelloides* CBS 108.16). In each case, total RNA (20 μ g per lane) was isolated from cultures grown in nitrogen-limited medium and harvested at 24, 48 and 72 h. It was hybridized with ME gene-specific probes prepared by RT-PCR. The RNA loading was standardized by ethidium bromide-stained gels prior to Northern blotting.

Fig. 5. Growth performance, lipid accumulation and ME activity of: *Mc. circinelloides* R7B wild-type (hatched bars), transformant *Mucor-malEMc* (black bars), and transformant *Mucor-malEMt* (open bars). Each strain was grown in a fully controlled, stirred 5 l fermenter using nitrogen-limited Kendrick medium. (a) Glucose in medium; (b) biomass; (c) ME activity in cell-free extracts; (d) lipid content of cells. In each case, ammonia (from 2 g ammonium tartrate l⁻¹) had been exhausted from the medium by 24 h, so that lipid accumulation would have been initiated ~2–4 h prior to this.

temporal difference in the persistence of ME activity, previously reported in the wild-type strains of *Mt. alpina* and *Mc. circinelloides* after nitrogen exhaustion (see Wynn *et al.*, 1999), is probably due to the different cellular half-lives of the respective ME proteins.

The downstream flanking regions of *malEMt* and *malEMc* were examined. No mitochondrial leader sequence was found from the deduced amino acids of either gene. The results of a BLAST search with GenBank showed high homology to other cytosolic ME genes (data not shown, but see Results for references). Therefore, these two MEs are presumptive cytosolic enzymes.

A single gene copy number of *malEMt* and *malEMc* in *Mt. alpina* and *Mc. circinelloides*, respectively, suggests that at least some of the multiple ME isoforms noted in both these fungi (Song *et al.*, 2001; Zhang, 2005) are encoded by different genes. Thus, the sequence of *malEMc* in this study is completely different from that of isoform II (*mce1*) from the same *Mc. circinelloides* strain that encodes a mitochondrial ME (Li *et al.*, 2005; and see Fig. 2). With isoforms III and IV (see Introduction), it is considered that both arise from the gene that has been cloned in this work; isoform IV, which appears later than isoform III (see Fig. 6), is probably formed by post-translational modification.

The process of lipid accumulation in oleaginous micro-organisms, whereby the lipid content may reach 70 %, and possibly higher, is still imperfectly understood. Although it has long been established that the build-up of lipid storage reserves, mainly in the form of triacylglycerols, requires the presence of ACL to generate acetyl-CoA in the cytosol (Botham & Ratledge, 1979), the activity of this enzyme does not correlate with the amount of lipid that a particular micro-organism may accumulate (Boulton & Ratledge, 1981; Ratledge & Gilbert, 1985; Wynn *et al.*, 1998). Indeed, a feature of lipid storage in micro-organisms, and also in plants, is that the maximum amount of lipid that can be stored in an individual species appears to be under genetic control in that there seems to be an absolute ceiling beyond which further increases in lipid content cannot proceed. The exceptions to this are some oleaginous yeasts, which accumulate lipid up to 70 % and beyond, and in which the maximum content is probably limited by the physical capacity of the cell to accommodate large oil droplets, bearing in mind that such cells still contain other organelles. In such organisms, all

general rule that a sequence in an intron contains more A + T than G + C.

The results of the Northern blot analysis suggest a similar manner of transcription of both ME genes. Therefore, the

Fig. 6. Activity staining of ME during growth of *Mc. circinelloides* wild-type (Wild) and transformants (see Fig. 5). Cell-free extracts were prepared from the wild-type and two transformant cultures, grown on nitrogen-limited medium for 24, 48, 72 and 96 h (see Fig. 5), then run on a native PAGE gel. ME activity was detected using the activity stain of Chang *et al.* (1991), which employs malate and NADP⁺ with coupling to Nitro Blue tetrazolium and overnight incubation at room temperature. (There was no activity when malate or NADP⁺ was omitted, or NAD⁺ was used in place of NADP⁺.) The two isoforms of ME are considered to arise from the same gene by post-transcriptional modification of isoform III to isoform IV.

enzyme activities that participate in glycolysis and lipid biosynthesis, including ME, remain active throughout the lipid-accumulation phase (Evans & Ratledge, 1983).

Whilst various proposals have been made to determine the rate-limiting step in both micro-organisms and plants (Ratledge & Wynn, 2002; Ohlrogge & Jaworski, 1997; Ramli *et al.*, 2005), none of the possible enzymes involved in either acetyl-CoA generation or fatty acid synthesis appears to have been vindicated by subsequent cloning experiments (Ohlrogge & Jaworski, 1997; Rangasamy & Ratledge, 2000). In our detailed biochemical analysis of lipid accumulation in *Mc. circinelloides*, which has been used commercially to produce an oil rich in γ -linolenic

acid (GLA; 18:3 n-6), and also in *Mt. alpina*, currently used to produce arachidonic acid (20:4 n-6), we had identified ME activity as the most likely rate-limiting step in this process (Wynn *et al.*, 1999, 2001; Song *et al.*, 2001). ME, through its oxidative decarboxylation of malate to pyruvate, provides NADPH which is essential for biosynthetic reactions. Although other NADPH-generating enzymes exist [glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (these two enzymes are involved in the initial reactions of the pentose phosphate cycle) and NADP⁺-dependent isocitrate dehydrogenase], their activities do not correlate with either the onset of lipid accumulation or its cessation. Of all the enzyme activities that have been monitored in both these fungi, only that of ME shows a close correlation with lipid accumulation. When ME activity ceases, so does lipid accumulation, whether it is at the limit of about 25 % cell dry weight for *Mc. circinelloides* or at ~40 % for *Mt. alpina* (Wynn *et al.*, 1999). Further, when ME activity was inhibited by sesamol, derived from sesame seed oil, lipid accumulation in *Mc. circinelloides* dropped from 25 to 2 % of the biomass, without any effect on cell growth (Wynn *et al.*, 1997). Thus, although the general view would suggest that there is a common pool of NADPH within a cell, our previous results would refute this, at least in the fungi we have studied, and have strongly suggested a positive linkage of ME activity with fatty acid biosynthesis (Ratledge & Wynn, 2002; Ratledge, 2004).

In this present work, we have now shown that lipid production can be increased substantially by placing the gene encoding ME [either isoforms III/IV from *Mc. circinelloides* (Song *et al.*, 2001) or its major isoform in *Mt. alpina* (Zhang, 2005)] under the control of a constitutive promoter. The transformed cells had a lipid content that was 2.5-fold that of the original parent, although it should be noted that the parent strain had a maximum lipid content of only 12 %; this was about half

Table 1. Lipid content and major fatty acyl constituents of *Mc. circinelloides* original wild-type and two transformants expressing an ME gene

The lipid in each of the three cultures, grown for 96 h (as described in Fig. 5), was extracted and the fatty acids (as methyl esters) analysed as described in Methods. Data are presented as mean values from a duplicate experiment. In each case, small amounts (<1 %) of 12:0, 14:0 and 16:1 fatty acids were also noted.

Lipid content	Strain		
	Wild-type	MtmalEMt	McmalEMc
Lipid (% w/w)	12.1 ± 0.6	28.6 ± 0.9	30.2 ± 0.8
Fatty acyl group			
16:0	20.7*	20.7	19.6
18:0	4.8	2.6	3.7
18:1 n-9	30.9	29.8	29.9
18:2 n-6	19.0	15.0	19.3
18:3 n-6	23.2	30.1	26.1

*Relative percentage (w/w) of the total fatty acyl groups.

that of the strain we had originally used, but the latter had had no prior genetic work carried out with it and so was inappropriate for genetic manipulation. Simultaneous with the substantial increase in lipid accumulation was a slight but definite overall increase in the GLA content of the lipid, indicating that the increased activity of ME was probably also generating NADPH for fatty acid desaturases (see Kendrick & Ratledge, 1992). Thus, increased ME activity led not only to an increased lipid content of the cells but also to an increased fatty acid desaturase activity.

It is, however, clear that there are still factors that are limiting the continued activity of ME even though it is now being expressed quasi-constitutively. Although we achieved higher expression of the enzyme, and consequently a higher activity than before, ME activity still declined after the exhaustion of nitrogen from the culture medium (see Fig. 5), in spite of the continued formation of the mRNA for ME (see Fig. 4). It is a prerequisite of lipid accumulation that nitrogen (or some nutrient other than carbon) be exhausted so that cell proliferation is stopped but carbon assimilation still continues. Lipid accumulation is then not so much an increased activity of appropriate lipid-synthesizing enzymes, but arises because other cell activities, including multiplication, have ceased or substantially declined. We have previously shown that during the lipid-accumulation phase in *Mc. circinelloides*, there is a change in ME from isoform III to isoform IV (Song *et al.*, 2001), which is possibly due to the removal of a short peptide sequence from the protein (Z. Bing & C. Ratledge, unpublished work), but with the overall consequence that ME activity gradually declines and eventually the enzyme ceases to be functional. This was again noted with both the transformants: ME activity still substantially declined. One possible explanation for this is that there could be an enzyme that specifically degrades ME (an ME-converting enzyme?) once the cells are nitrogen limited and have commenced lipid storage.

Lipid accumulation thus can only continue for as long as ME continues to be active. The transformed cells were still limited in their capacity to accumulate lipid because of the continuing inactivation (conversion?) of ME. They were still some way off what would be regarded as a physical limit of lipid production. It may be conjectured that if ME could be engineered to resist degradation (or if the gene for the suggested ME-converting enzyme could be deleted) then fungi such as *Mc. circinelloides* and *Mt. alpina* should be able to accumulate as much lipid as the most efficient oleaginous yeasts, in which ME does indeed remain fully active (Evans & Ratledge, 1983) and lipid can accumulate to very high levels (>70 %).

Whether increasing ME activity in other systems, such as plant and animal cells, would also lead to increased lipid accumulation is less certain. In plants, there is a wide range of functions for ME, which is regarded as a ubiquitous enzyme that is involved in a variety of different metabolic pathways, ranging from a role in photosynthesis to other

still unknown roles (Drincovich *et al.*, 2001). Thus, identifying the correct ME gene involved in generating NADPH specifically for fatty acid biosynthesis may not be an easy task. In animal cells, although ME has been recognized for some time as being important for generating NADPH for fatty acid biosynthesis (e.g. see Castelein *et al.*, 1994; Hillgartner & Charron, 1998; Sourdioux *et al.*, 1999; Ceddia *et al.*, 2000), it is not the only provider: both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase also provide ~50 % of the NADPH for fatty acid biosynthesis (Rognstad & Katz, 1979; Shimomura *et al.*, 1998). These two enzymes are part of the pentose phosphate pathway, and like ME, remain active during lipid formation. They only appear to be repressed by starvation of the animal (Hillgartner & Charron, 1998). However, in oleaginous micro-organisms, the pentose phosphate pathway is repressed during lipid accumulation (Evans & Ratledge, 1983, 1984), as its operation is not required to provide intermediates for protein and nucleic acid biosynthesis, both of which are stopped by the exhaustion of nitrogen from the culture medium at the start of lipid accumulation. Such conditions, of course, could not apply to animals.

Thus, it is probably only in micro-organisms that ME assumes a unique role in fatty acid biosynthesis that cannot be replaced by another enzyme. This uniqueness would also seem to apply to the formation of unsaturated fatty acids (Kendrick & Ratledge, 1992), which also requires NADPH and, being a membrane-associated activity, clearly needs a mechanism by which the highly water-soluble cofactor can be supplied to the fatty acid desaturases contained within the membranes of the endoplasmic reticulum. ME evidently fulfils this second role in addition to its role in fatty acid biosynthesis, as we have shown here.

Codicil

This paper concludes the work of C. R. on the biochemistry of lipid accumulation in oleaginous micro-organisms. Although further papers may be published on various other aspects of lipid formation in micro-organisms, this is the final work in which the key role of ME and its attendant gene expression will be investigated. Other interested researchers are therefore free to develop whatever aspect of these studies may seem attractive to them.

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