A *Saccharomyces cerevisiae* gene required for heterologous fatty acid elongase activity encodes a microsomal β-keto-reductase.

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Running Title: microsomal elongase β-keto-reductases
Abstract

A number of *Saccharomyces cerevisiae* membrane-bound oxidoreductases were examined for potential roles in microsomal fatty acid elongation, by assaying heterologous elongating activities in individual deletion mutants. One yeast gene, YBR159w, was identified as being required for activity of both the *Cenorhabditis elegans* elongase PEA1 (F56H11.4) and the *Arabidopsis thaliana* elongase FAE1. Ybr159p shows some limited homology to human steroid dehydrogenases and is a member of the short chain alcohol dehydrogenase superfamily. Disruption of YBR159w is not lethal, in contrast to previous reports, though the mutants are slow-growing and display high temperature sensitivity. Both Ybr159p and an Arabidopsis homologue were shown to restore heterologous elongase activities when expressed in *ybr159Δ* mutants. Biochemical characterisation of microsomal preparations from *ybr159Δ* cells revealed a primary perturbation in β-keto acyl reduction, confirming the assignment of YBR159w as encoding a component of the microsomal elongase.
Introduction

Unsaturated fatty acids are essential cellular constituents, serving not only as structural components of membranes but also as bioactive metabolites. One important class of these lipids is collectively known as polyunsaturated fatty acids (PUFAs); these are defined as fatty acids of 18 carbons or more (C18+) which contain two or more double bonds\(^1\). In mammals, 20 carbon (C\(_{20}\)) PUFAs have been shown to be the biological precursors of a group of molecules called the eicosanoids, which includes the prostaglandins, leukotrienes and thromboxanes\(^2\). The eicosanoids have roles in inflammation responses, as well as cardiac and reproductive function. This obvious importance of PUFAs has resulted in considerable interest in the characterisation of their biosynthetic pathway\(^3\) and we have identified a new class of fatty acid desaturases required for PUFA biosynthesis which contain an N-terminally fused cytochrome b\(_5\) domain\(^4\).

More recently, we have also identified a component of the PUFA fatty acyl chain elongation system, which when heterologously expressed in yeast, directs the C\(_2\) elongation of the C\(_{18}\) PUFA \(\gamma\)-linolenic acid (18:3 n-6; GLA) to the C\(_{20}\) PUFA di-homo-\(\gamma\)-linolenic acid (20:3 n-6; DHGLA)\(^5\). This component (\(C.\) elegans ORF F56H11.4, designated PEA1 for polyunsaturated fatty acid elongating activity\(^5,6\)) shows some limited homology to the yeast ELO gene family, which have been shown genetically to be required for the synthesis of saturated medium and very long chain fatty acids\(^7,8\). Although the precise biochemical function of the \(C.\) elegans PUFA-elongating activity PEA1 and the polypeptides encoded by the ELO genes remains to be elucidated, it is generally considered that they serve as condensing enzymes\(^5,6\). The steps in the biosynthetic pathway of C\(_{20}\) PUFAs are indicated in a generalised scheme (Figure 1), with the key step of C\(_2\) elongation of C\(_{18}\) substrates highlighted.

The biosynthesis of C\(_{20}\) fatty acids has also been observed in higher plants, though the elongated fatty acids are quite distinct to those observed in mammals. Although C\(_{18}\) PUFAs
such as α-linolenic acid and linoleic acid accumulate to high levels in plants, the presence of C_{20} PUFAs has not been observed\textsuperscript{1}. However, some higher plants, most notably members of the Brassicas such as oil seed rape and Arabidopsis, synthesise C_{20} and C_{22} monounsaturated fatty acids (MUFAs)\textsuperscript{9}. These C_{20+} fatty acids are the product of (several cycles of) C_{2} fatty acyl elongation of a monounsaturated C_{18} substrate, oleic acid (18:1Δ^\text{9}). The biosynthesis of higher plant C_{20/22} MUFAs has been shown to require a single gene, FAE1 (fatty acid elongation) which encodes a putative condensing enzyme\textsuperscript{10}. The Fae1p condensing enzyme displays specificity only for saturated and monounsaturated fatty acids\textsuperscript{9,10}. Importantly, although Fae1p shows some limited homology to analogous condensing enzymes such as chalcone and stilbene synthases, it displays no similarity to the Elo protein family; conversely no homologs of Fae1p are present in yeast.

Fatty acid elongation is carried out by a microsomal “elongase” which consists of four distinct enzymatic reactions\textsuperscript{11}. In sequential order, these are a condensation reaction between an CoA-esterified fatty acyl substrate and malonyl CoA, β-keto-reduction, dehydration and a final enoyl reduction. It has been hypothesised that the specificity of any particular elongation reaction is conferred through the selectivity of the first condensation step. Conversely it is believed that the three other components (two reductases and a dehydratase) are common to all microsomal fatty acyl elongases, and have no particular substrate specificity\textsuperscript{11}. This is given credence by the observation that heterologous expression in yeast of the plant Fae1p condensing enzyme successfully reconstitutes a functional C_{20+} MUFA-specific elongase\textsuperscript{9}. Similarly, we have also observed effective reconstitution of the PUFA-specific elongase (via by heterologous expression of the \textit{C. elegans} Pea1p in yeast)\textsuperscript{5}, even though yeast has no endogenous capacity for either of these particular biosynthetic reactions.
We have hypothesised that these heterologous elongating activities function by “hi-
jacking” endogenous microsomal elongases, which in the case of yeast, are primarily
synthesising the C_{20-26} saturated fatty acid components of sphingolipids\(^5\). This results in the
presumptive redirection of the three endogenous components (reductases and dehydratase)
towards non-native substrates. Until very recently, little was known about the identity of
these other three enzyme activities and their precise contribution(s) to the activity and
specificity of microsomal fatty acid elongases. Kohlwein et al.\(^{12}\) characterised the \(TSC13\)
gene, which is one of a number of genes identified in a screen for temperature sensitive (ts)
mutants with defects in sphingolipid synthesis\(^{13}\). Mutants with ts alleles of \(TSC13\) displayed
phenotypes similar to the \(elo2\Delta\) and \(elo3\Delta\) mutants, including the accumulation of high levels
of long chain bases, the accumulation of ceramides with chain lengths of less than 26 carbons,
and a deficiency in VLCFA synthesis\(^{12}\). Microsomal fatty acid elongation assays revealed
that the \(tsc13\) mutation caused the accumulation of trans-2,3- and 3-hydroxy-acyl
intermediates; these observations, taken together with the homology between Tsc13p and
steroid-5-\(\alpha\)-reductase, are consistent with the \(TSC13\) gene encoding the enoyl reductase
component of the microsomal elongase. Furthermore, epitope-tagged Tsc13p was shown to
co-immunoprecipitate with the presumptive condensing enzymes Elo2p and Elo3p\(^{12}\).
Interestingly, a precise ER location for Tsc13p, enriched at sites of vacuole-nuclear envelope
interaction was observed. \(TSC13\) is essential, as is expected for a gene encoding a non-
redundant fatty acid elongating activity\(^{12}\).

As part of our studies on microsomal fatty acid elongation, we have sought to identify
other enzymatic components of the fatty acid elongase. To that end, we screened yeast
knockout mutants for loss-of-function of our heterologous PUFA elongating activity PEA\(^{15}\).
Using this approach we identified a previously functionally uncharacterised gene required for
the reconstitution of heterologous elongase activity.
Materials and Methods.

Cloning of putative β-ketoacyl reductases in a Yeast Expression Vector.

The Saccharomyces cerevisiae ORF encoded by YBR159w was cloned by PCR into the pESC-TRP vector (Stratagene). A crude DNA extract was prepared using the reference strain W303-1A. 1 ml of an overnight culture in rich medium (YPD) was centrifuged at 3000 g for 5 minutes at room temperature and the cells resuspended in 0.5 ml TE. This suspension was boiled for 10 minutes and the extract centrifuged at 10,000 g for 5 minutes. 1 µl of the supernatant was used for PCR amplification using primers YBR159.for (5’-GCGGGATCCACCATGACTTTTATGCAACAGC-3’) and YBR159.rev (5’-GCGGGATCCCTATTCCTTTTTAACGTCT TGC-3’). The amplified sequence was then restricted using BamHI and KpnI (underlined in the forward and reverse primers respectively) purified using the Qiagen PCR purification kit and ligated into BamHI/KpnI cut pESC-TRP plasmid vector (Stratagene). An Arabidopsis thaliana EST (GenBank Accession Number: AA10E08; kindly provided by Prof. H. J. Bohnert, University of Arizona) derived from gene F12A21.31 was used as template DNA for PCR amplification using primers At159.for (5’-GCGGGATCCACCATGGAGATCTGCACTTACTTC-3’) and At159.rev (5’-GCGGGATCCATTCTTTCTTCATGGAGTC-3’). The amplified sequence was then restricted using BamHI and XhoI (underlined in the forward and reverse primers respectively) and cloned into BamHI/XhoI cut pESC-TRP as described above.

Functional Characterisation in Yeast.

ORFs encoding putative β-ketoacyl reductase activities and elongase or desaturase constructs were introduced in Saccharomyces cerevisiae using a lithium acetate based method. Expression of the transgenes was induced by addition of 2% (w/v) galactose in the presence
or in the absence of exogenously supplied fatty acid substrates as described previously\textsuperscript{15}. The mutant strain used in this study was CEN.RO16; Mat a/α; ura3-52/ura3-52; his3Δ1/his3Δ1; leu2-3_112/leu2-3_112; trp1-289/trp1-289; \textit{ybr159Δ::HIS3/YBR159w}, obtained from EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). This strain was transformed with a pYES2 construct containing the \textit{C. elegans} PUFA elongating activity PEA1 (F56H11.4) and sporulated in SPM liquid media as described\textsuperscript{16}. After sporulation, asci were digested with β-glucuronidase (Sigma) and the tetrads dissected using light microscopy and a micromanipulator as described\textsuperscript{17}. Separated ascospores were grown on YPD for up to 2 weeks at 22°C and the mating type of each haploid colony tested using two yeast strains, a \textit{sst1} and α \textit{sst2}\textsuperscript{18}. “Wild type” (Mat a or α; ura3-52; his3Δ1; leu2-3_112; trp1-289) and mutant spores (Mat a or α; ura3-52; his3Δ1; leu2-3_112; trp1-289; \textit{ybr159Δ::HIS3}) were identified by replica plating on SD lacking histidine. This was also confirmed by PCR using primers 159\_prom.for (5’-CGGATTTGGAAGTCCTTTATAG-3’) and 5’\_his3.rev (5’-CGCTTTACTAGGGCTTTC TGC-3’). Wild type and mutant spore colonies containing the PUFAs-elongase construct in pYES2 were selected by replica plating on SD lacking uracil.

\textit{Fatty Acid Analysis}

Total fatty acids extracted from yeast cultures were analysed by gas chromatography (GC) of methyl ester derivatives. Lipids were extracted and transmethylated with methanolic HCl. Fatty acid methyl esters (FAMEs) were analyzed as described before\textsuperscript{19}.

\textit{GC-MS Analysis}

Induced peaks were characterised using GC-MS (Kratos Analytical instruments MS80RFA) operating at an ionisation voltage of 70 eV with a scan range 40-500 Da and as described before\textsuperscript{19}.
Assay of Elongase Activity

Elongase Assays. Microsomes were prepared from the wild-type or ybr159∆ mutant cells as has been previously described. Total elongase activity was measured in a volume of 200 µl containing 50 mM Tris, pH 7.5, 1 mM MgCl₂, 150 µM triton X-100, 1 mM NADPH, 1 mM NADH, 10 mM β-mercaptoethanol, 40 µM palmitoyl-CoA, 60 µM 2[^14]C]-malonyl-CoA (0.05 µCi/ml) at 37°C. The reaction was initiated by the addition of 0.4 mg of microsomal protein. Protein concentrations were determined using the Biorad protein assay reagent (Bio-Rad Laboratories). For assays of only the condensing activity, the NADPH and NADH were omitted. At various times (0.2, 1 or 5 minutes) the reaction was terminated by adding 200 µl of 5 M KOH/10% MeOH and heating at 80°C for 1 h. Following addition of 200 µl of 10 N H₂SO₄, fatty acids were recovered by two 1.5 ml extractions into hexane. The extracted fatty acids were resolved by silica gel TLC using hexane:diethyl ether:acetic acid (30:70:1) as the developing solvent and detected and quantified using a PhosphorImager SI (Molecular Dynamics, Inc.).
Results

In an attempt to identify gene(s) encoding the β-keto reductase of the microsomal elongase, we searched the complete genome sequence of the yeast *Saccharomyces cerevisiae* (predicted to encode between 5000 and 6000 polypeptides) for protein sequences likely to encode oxidoreductases. This was carried out either on a homology basis to known oxidoreductases or via the presence of the diagnostic NADH binding motif. Since this generated over 200 candidates, the search was refined to exclude proteins already clearly functionally characterised, as well as cytosolic oxidoreductases. By excluding predicted ORFs which lacked a canonical dilysine ER-retention motif, the search was further refined to identify predicted oxidoreductases likely to have a transmembrane topology and to be located in the ER. This approach allowed us to consider a small number (~10) of genes for functional characterisation as potential microsomal fatty acyl elongase components (listed in Table 1).

A number of these selected oxidoreductases ORFs had previously been shown by high-throughput deletion analysis to be non-essential for viability. Yeast mutants in which the non-essential candidate genes were disrupted were assayed for any loss of ability to carry out heterologous PUFA elongation. Thus, a galactose-inducible, URA3-marked plasmid carrying the *C. elegans* PEA1 PUFA elongating activity was transformed into these mutant strains, and the transformed yeast cells were assayed for their ability to direct the C₂ elongation of GLA, forming the basis of a “loss-of-function” screen.

Characterisation for loss-of-function in haploid knockout mutants of the eight non-essential oxidoreductases (Table 1) revealed no alteration in their ability to reconstitute the heterologous PUFA elongase and redirect the elongation of C₁₈ PUFAs. Although the synthesis of very long chain fatty acids is essential for yeast cell viability, previous directed (EMS) mutant screens for defects in fatty acid elongation only identified the *ELO1* gene as a potential elongase component, implying that other elongase genes are either essential or
display functional redundancy\textsuperscript{7,8,23}. To address the possibility that the (annotated as) essential gene YBR159w encoded an oxidoreductase component of the microsomal elongase, a diploid yeast strain heterozygous for disruption of YBR159w was transformed with a plasmid containing the PEA1 ORF. Following sporulation and tetrad dissection, the spores were allowed to germinate on YPD media. Using this approach, we observed that the \textit{ybr159Δ} haploid deletion was in fact viable, though PCR confirmed the insertional disruption of this oxidoreductase ORF in mutant haploid cells (data not shown). When compared with wild type spores isolated from the same tetrad, spores containing the disrupted gene showed a very reduced growth rate, with small colonies appearing usually only after 10 days incubation at 22ºC.

An initial study described deletion of YBR159w (in strain ENY.MR17) as resulting in poor growth (only at low temperatures) with a pseudo-hyphal phenotype\textsuperscript{24}, though in a subsequent study it was reported that YBR159w was an essential gene in the CEN.PK2 background\textsuperscript{25}. In our current study, we used the same parental diploid heterozygote as used in the latter study (CEN.PK2 strain background), though after sporulation the haploid \textit{ybr159Δ} mutants were viable even at 30ºC. Indeed, although it took about ten days for mutant spores to germinate and to form a colony after meiosis (see above), \textit{ybr159Δ} cells are able to grow at 30ºC in rich medium (in the absence of fatty acid supplements), although at a slower rate than wild type (Fig 2). Interestingly, cultivating \textit{ybr159Δ} cells in a media supplemented with medium and long chain fatty acids did not improve the growth rate (Data not shown). However, we observed that the \textit{ybr159Δ} mutant displayed temperature-sensitivity when transferred to 37ºC. We also examined the phenotypic appearance of the CEN.PK strain \textit{ybr159Δ} mutant cells and observed the previously reported (for strain ENY.MR17) pseudo-
hyphal growth. However, genetic analysis indicates that this phenotype is unlinked to the ybr159Δ mutation (TD, unpublished observations).

The oxidoreductase Ybr159p is specifically required for heterologous elongation activity in S. cerevisiae

The ability of the heterologous elongating activity Pea1p to function in the ybr159Δ deletion was tested by galactose-induction as described above. Wild-type haploid colonies displayed PUFA-elongating activity resulting in the conversion of 18:3, n-6 (GLA) into 20:3, n-6 (DHGLA), whereas deletion mutant haploids completely failed to elongate heterologous C₁₈ PUFAs (Fig 3, top panel). We tested the galactose-inducibility of other pYES2-directed enzyme activities in this haploid knockout strain, to assess the possibility of pleiotropic effects on either galactose uptake or lipid metabolism. However, when two other (heterologous) enzymes of the PUFA biosynthetic pathway (borage Δ⁶-desaturase, C. elegans ω³-desaturase; see also Figure 1) were tested in this mutant background, they displayed unaltered activities when compared with wild-type yeast (Table 2). Northern blot analysis of mutant cells expressing the heterologous elongase revealed no alteration in transcript abundance when compared with wild-type (data not shown). Thus, deletion of YBR159w had a specific effect on the activity of the heterologous PUFA elongase reconstituted by expression of PEA1. This was given additional weight by our observation that YBR159w was also required for the activity of the Arabidopsis microsomal elongase condensing enzyme FAE1; expression of Fae1p in ybr159Δ haploid strains failed to accumulate C₂₀+ MUFAs (Figure 3, bottom panel).
Several ORFs related to YBR159w are present in S. cerevisiae and in other yeast, plant and animal genomes.

Examination of the deduced amino acid sequence encoded by YBR159w indicated that the predicted polypeptide (of 347 amino acids) showed some similarity to human estradiol 17-β-hydroxysteroid dehydrogenase (32% similarity). For this reason, it has recently been suggested that the enzyme encoded by YBR159w could be responsible for steroid dehydrogenase activities observed *in vitro* in yeast extracts. However, no sequences homologous to YBR159w could be detected by PCR and northern hybridisation in the mesophilic yeasts *Candida tropicalis* and *Cryptococcus tsukubaensis* even though steroid dehydrogenase activity was observed. These observations have led the authors to speculate that the Ybr159p oxidoreductase does not function as a steroid dehydrogenase; this is in agreement with our present study. Presumptive orthologs of Ybr159p are also present in fission yeast (43% similarity), *Drosophila melanogaster* (34% similarity), *C. elegans* (30% similarity) and Arabidopsis (30% similarity) (Figure 4A). Not only do all these sequences contain the diagnostic NADH binding motif, they also share a number of conserved residues, in particular the catalytically essential (for estradiol-17-β-hydroxysteroid dehydrogenase) motif Y-X$_3$-K. There are also canonical dilysine ER-retention motifs present in both Ybr159p and the *Arabidopsis* ortholog, consistent with these proteins’ predicted multiple (presumptively endoplasmic reticulum) membrane-spanning topology.

When the polypeptide sequence of Ybr159p was used to search the complete yeast genome sequence, several related, but distinct, ORFs were detected. These included Ymr226p (which shows some homology to insect short chain alcohol dehydrogenase; 33% similar), Ayr1p (1-acyl-dihydroxyacetone phosphate reductase: 24% similar) and Yir036p (which shows homology to 7 α-hydroxysteroid dehydrogenase; 23%) (Fig 4B). Individual deletion analysis of these three ORFs had previously indicated that none of these genes encode essential
proteins. More importantly, individual deletion of any of these ORFs (YMR226c, AYR1/YIL124w, YIR036c) did not alter the activity of the heterologous PUFA elongase PEA1 as determined by our loss-of-function assay (data not shown; see also Table 1 for summary).

*ybr159Δ mutants can be complemented by a homologous ORF from A. thaliana.*

Further confirmation of the role of Ybr159p in heterologous microsomal elongation activity was obtained by rescue of either Pea1p or Fae1p (PUFA or MUFA, respectively) elongating activities by episomal co-expression of either the wild type YBR159w ORF from *S. cerevisiae* or a presumptive *Arabidopsis thaliana* homologue ORF (F12A21.31). In *ybr159Δ* cells, the galactose-induced co-expression of YBR159w with PEA1 resulted in restoration of 72% of C18 PUFA elongating activity, respectively, when compared to the activity of the same enzymes in wildtype cells (Table 3). When the presumptive Arabidopsis ortholog F12A21.31 was co-expressed instead of YBR159w, this resulted in a very similar restoration of PUFA elongation (Table 3); for that reason we hereafter refer to F12A21.31 as At-YBR159. Similarly, co-expression of YBR159w with FAE1 in *ybr159Δ* cells resulted in a 52% restoration of C18 MUFA elongation, while co-expression with At-YBR159 resulted in restoration of 42% of FAE1 activity, compared with wild type cells (Table 4).

Interestingly, episomal galactose-induced over-expression of YBR159w in wild type spore colonies (derived from the same tetrads which had yielded the *ybr159Δ* haploids) resulted in moderate increases in both PEA1 (PUFA) and FAE1 (MUFA) heterologous elongation activities, compared with the expression of these elongating activities in wild type cells (Tables 3 and 4). This suggests that, in contrast with native elongase systems, the condensation reaction may not be the only rate limiting activity for heterologous elongation in
yeast. Co-expression of the same elongating activities with At-YBR159 in wild-type yeast resulted in almost equivalent results, again demonstrating the functional equivalence of the Arabidopsis protein.

*ybr159Δ mutant cells have reduced endogenous fatty acid elongation activity.*

Ybr159p clearly plays a role in the fatty acid elongation mediated by the heterologously-expressed Fae1p and Pea1p activities in yeast. Based on its homology to the oxidoreductases, it was considered a good candidate for a β-keto-reductase, reducing the 3-keto intermediate formed by the condensing activity during each cycle of fatty acid elongation. To address how elongation is affected in the ybr159Δ mutant cells, microsomes were prepared and assayed for elongase activity *in vitro*. The elongation cycle initiates with the condensation of malonyl-CoA with an acyl-CoA (e.g., palmitoyl-CoA) to form a 3-keto-acyl-CoA intermediate. Omitting pyridine nucleotide from the assay mix prevents the reduction of the 3-keto-acyl-CoA intermediate, and thereby allows the first step of elongation to be measured. The condensation activity measured over a time course of 5 minutes was very similar whether wild-type or ybr159Δ microsomes were used for the assay (Figure 5, compare lanes 4-6 and 10-12). However, when the time-course of the overall elongation reaction (with NADH/NADPH included) was conducted and the products were analysed by TLC using conditions that resolved the four intermediates of fatty acid elongation, differences between the wild type and ybr159Δ mutant were apparent. The only intermediate seen in the elongation catalysed by the wild-type microsomes was a small amount of 3-hydroxy intermediate in the 5 minute time point (Figure 5, lane 3) under conditions where a large amount of fully elongated product accumulated. In contrast, a large amount of the 3-keto
intermediate accumulated and the formation of the fully elongated product was greatly delayed during the elongation catalysed by the ybr159Δ mutant microsomes (Figure 5, lane 8 and 9). Based on the homology of Ybr159p to oxidoreductases, it may directly catalyse the reduction of the 3-keto intermediate that is formed in each cycle of elongation. However, in addition to the 3-keto intermediate, elevated amounts of the 3-hydroxy intermediate also accumulated (compare the difference in the ratio of this intermediate to the fully elongated product in the mutant to that in the wild type, Figure 5, lane 3 vs. lane 9). While the accumulation of the 3-keto intermediate of elongation would be consistent with Ybr159p functioning as a β-ketoreductase, it is not clear why the 3-hydroxy intermediate would also accumulate in the mutant (see the Discussion for possible explanations). In addition, while Ybr159p is not essential for viability, VLCFAs are; therefore, if Ybr159p is a β-ketoreductase activity that functions in elongation, there must be functionally redundant activity; this is consistent with the non-lethality of the disruption of this gene.

Discussion.

In this study, we report the identification of a yeast gene required for the reconstitution of a heterologous microsomal fatty acid elongase. This gene, identified as YBR159w is predicted to encode an integral membrane protein, most likely located in the ER. The encoded protein shows some limited homology to mammalian steroid dehydrogenases, most notably estradiol 17-β-hydroxysteroid dehydrogenase, and is a member of the short chain dehydrogenase superfamily (Sanger Protein Family Database ID: Pfam00106). Interestingly, the Arabidopsis homologue of Ybr159p functionally characterised by us in this study was previously annotated by Nikolau and colleagues as being an ortholog of the maize Glossy8 gene30. Transposon-induced mutations in this maize gene result in defects in the synthesis of the cuticular waxes deposited on the outer epidermis of the plant’s cells, giving the mutant
plant a characteristic “glossy” appearance\textsuperscript{30}. The maize \textit{glossy8} locus was cloned via transposon inactivation-tagging and, although not characterised biochemically, was hypothesised (on the basis of homology to other dehydrogenases) to encode a \(\beta\)-ketoacyl-reductase\textsuperscript{30}. Homozygous \textit{glossy8} maize mutants display decreased levels of \(C_{24+}\) components of wax esters but are viable indicating either gene redundancy or the presence of distinct elongases for fatty acid and wax synthesis. A partial-length cDNA clone from Arabidopsis (GenBank Accession number U89512) was also identified in that study as being a likely ortholog of maize Glossy8. That Arabidopsis transcript is derived from F12A21.31, the gene identified by us as encoding a functional homolog of the yeast microsomal \(\beta\)-keto-reductase Ybr159p. Based on the data presented in this paper, we believe it is now possible to assign the function of \(\beta\)-keto-reductase to the maize Glossy8 locus.

Previous assessments of the requirement of YBR159w for yeast viability yielded slightly contradictory data, with the gene being described as essential in the CEN.PK2 background but viable at reduced temperatures in the ENY.MR17 background. One explanation for the discrepancy in previous viability data may be explained by our observation of a long “lag” period in the initial growth of newly isolated haploid mutants. This initial slow growth may be related to some form of adaptive response to the loss of this microsomal elongase component. After this “adaptation” the mutant spore colonies formed are viable in the absence of fatty acid supplement, although growing at a much slower rate than wild type cells (Figure 2). In that respect it may be analogous the adaptation of \textit{srp54A} null mutants, in which the loss of a component of the ER protein targeting machinery is bypassed through an adaptive mechanism\textsuperscript{31}.
Mutagenic approaches to the identification of genes involved in fatty acid elongation have previously only identified one component, Elo1p, which is believed to be a condensing enzyme. The failure to recover mutants in the other enzymatic reactions which comprise the elongase system have been interpreted as indicating either functional redundancy (as exemplified in the case of the ELO2/3 genes) or lethality on disruption. The essential nature of the activity of the microsomal elongase for yeast cell viability is clear from the synthetic lethality of elo2elo3 mutants, as well as the requirement for a functional copy of the TSC13 gene, which encodes the enoyl reductase. Our observation that YBR159w, a non-essential gene, is required for microsomal elongase activity is intriguing, especially because of the lack of (functionally redundant) candidate homologs in the yeast genome. It is also clear that the lethality (or not) on the loss of YBR159w is strain-dependent; our current studies in the CEN.PK2 background indicate the viability of ybr159Δ mutants. Whilst there are no obvious homologs of Ybr159p, it is possible that related, ER co-located oxidoreductases (i.e. paralogs of Ybr159p) are capable of (inefficiently) metabolising 3-ketoacyl-CoA intermediates. This could explain the viability of the ybr159Δ mutant, as well as the observed pronounced reduction (but not ablation) in elongase activity.

The experiments presented here suggest that Ybr159p is the β-keto-reductase activity of the microsomal fatty acid elongating system. Consistent with this proposal, the in vitro elongation assays reveal an accumulation of the 3-keto-fatty acyl elongation intermediate. The accumulation of 3-hydroxacyl-CoA intermediates in the flux through the elongase of ybr159Δ is equally intriguing. This could be due to a perturbation of the physical interactions between the various distinct enzymatic components as a result of the loss of Ybr159p. As such, this would give further credence to the hypothesis that the microsomal elongase is likely to function as a large multimeric protein complex. In that respect, proximal physical
interactions between other microsomal elongase components such as Tsc13p and Elo2p/Elo3p have previously been demonstrated\cite{12}.

**Conclusions.**

In this study we have used a "loss-of-heterologous-function" screen to genetically identify a component of the microsomal fatty acid elongase. Biochemical characterisation confirms the identification of the encoded polypeptide as the \(\beta\)-keto-reductase. As such, this is the first report of the identification of a gene encoding this microsomal enzyme. Moreover, overexpression of the \(\beta\)-keto-reductase can result in enhanced activity of (heterologous) elongases, challenging the concept that the condensing enzyme is the rate-limiting step in fatty acyl elongation.

**Acknowledgements.**

IACR-Long Ashton Research Station receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK. This work was also supported by a grant (to TMD) from the National Science Foundation.

**Footnotes.**

**Abbreviations:**

GLA: \(\gamma\)-linolenic acid; MUFA: monounsaturated fatty acid; ORF: open reading frame; PUFA: polyunsaturated fatty acid
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Figure Legends

Figure 1.

*Generalised pathway for the biosynthesis of polyunsaturated fatty acids*

A generalised scheme of the enzymes responsible for aerobic desaturation and elongation of fatty acids is shown. The reaction directed by the polyunsaturated fatty acid elongase PEA1, resulting in the production of C$_{20}$ PUFAs is boxed. The yeast *S. cerevisiae* does not synthesise PUFAs, serving as a convenient host for “gain-of-function” screening.

Figure 2.

*Wild-type and ybr159Δ mutant cells display different growth rates in YPD at 30°C.*

Liquid cultures in YPD were inoculated with wild type and mutant cells, picked from YPD plates conserved at 4 °C, and incubated for up to 72 h at 30 °C. Cell growth was monitored by measuring the optical density of the cultures at 600nm.

Figure 3.

*Analysis of fatty acid elongation activity in ybr159Δ yeast.*

Chromatograms of fatty acid methyl esters (FAMEs) prepared form wild type (WT) and mutant (ybr159Δ) yeast expressing either *C. elegans* PUFA elongating activity PEA1 (top panel) or *A. thaliana* MUFA elongating activity FAE1 (bottom panel). γ-Linolenic acid (18:3 Δ6,9,12) was supplied as exogenous substrate to cells expressing PEA1. Fatty acid elongation was detected by flame ionization, with peak identification by subsequent GC-MS. In wild type cells the expression of PEA1 or FAE1 resulted in the production of 18, 20 and 22 carbon elongation products (peaks indicated by arrows). In *ybr159Δ* cells elongation activity was completely absent. *: The expression of FAE1 in wild type yeast cells also resulted in the accumulation of 14 carbon saturated and monounsaturated fatty acids.
Figure 4.

A. Sequence comparisons of *S. cerevisiae* gene product Ybr159p and homologues from other species.

SPAC4G9.15: putative short chain alcohol dehydrogenase from *S. pombe* (CAA93565); C56G2.6: possible estradiol 17-β-hydroxysteroid dehydrogenase from *C. elegans* (AAA64333); CG1444: putative oxidoreductase from *D. melanogaster* (BI370369); F12A21.31: putative β-keto acyl reductase from *A. thaliana* (AF361844); HSD17B3: 17-β-hydroxysteroid dehydrogenase from *H. sapiens* (NP_000188).

B. Sequence comparisons of Ybr159p and related sequences from *S. cerevisiae*:

Ymr226p (CAA90197); Yil124p (CAA86868); Yir036p (CAA86196). Potential membrane spanning regions were identified using the TopPred2 topology prediction server (available at [http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html](http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html)) and are indicated by black lines. The putative NADH binding motif [G(x)3GxG(x)3A(x)3A(x)2G] and the essential catalytic motif [Y(x)3K] are also indicated.

Figure 5:

*ybr159Δ* cells are deficient in fatty acid chain elongation.

Fatty acid elongation activity in wild-type (lanes 1-6) and *ybr159Δ* mutant (lanes 7-12) microsomes was compared using palmitoyl-CoA and radiolabeled malonyl-CoA as substrates. The assays were performed in the absence of NADH/NADPH (lanes 4-6 and 10-12) to measure condensation activity and in the presence of NADH/NADPH (lanes 1-3 and 7-9) to measure overall elongation activity. The reactions were stopped at the indicated times and the fatty acids were extracted and separated by TLC. The positions where the 3-ketostearate (3-Keto), 3-hydroxystearate (3-Hydroxy) and stearate standards (included on the
TLC and visualised by charring after exposure to the PhosphorImager screens) migrated are indicated.
Table 1.

Yeast oxidoreductases identified as candidate components of the microsomal elongase.

ORFs with unassigned biochemical function but displaying significant similarity to functionally characterised activities are indicated by *. YBR159w was previously reported as being essential, though results obtained in this study indicate that ybr159Δ mutant cells are actually viable. The activity of the heterologous elongating activity PEA1 in deletion mutant strains is indicated.

Table 2. Expression of desaturase genes in ybr159Δ yeast.

Fatty acid composition of ybr159Δ or wild type yeast transformed with either the borage Δ6-fatty acid desaturase (BorΔ6) or the C. elegans Δ3-fatty acid desaturase (FAT1) cloned into the pYES2 vector (Invitrogen) under the control of the GAL1 promoter. Linoleic acid (C18:2 Δ9,12) was added to the cultures before induction with galactose. Induced cultures were incubated at 22 ºC for 48 h and the fatty acid composition analysed as described in Materials and Methods. All values are expressed as mol. percentage of total fatty acids. And standard deviations are given. The percentage desaturation of substrates was calculated using the following formula: Product /(Substrate + Product)*100, Substrate and Product being the mol. percentage of LA and ALA or GLA, respectively.

Table 3. Rescue of PUFA elongation activity in ybr159Δ yeast. Fatty acid composition of mutant (ybr159Δ) or wild type (WT) yeast coexpressing PEA1 (cloned into pYES2) and either S. cerevisiae ORF YBR159w or AtYBR159, an homologue ORF from A. thaliana (both cloned into pESC-TRP). All episomal gene constructs were under the control of the GAL1 promoter. γ-Linolenic acid (18:3 Δ6,9,12) was supplied as exogenous substrate and the fatty acid composition of induced yeast was analysed as described for Table 2. The percentage
elongation of substrates was calculated using the following formula: Product / (Substrate + Product) * 100, Substrate and Product being the mol. percentage of 16:1Δ⁹ and 18:1Δ¹¹ or of GLA and DHGLA, respectively.

Table 4. Rescue of MUFA elongation activity in ybr159Δ yeast. Fatty acid composition of mutant (ybr159Δ) or wild type (WT) yeast coexpressing FAE1 and either S. cerevisiae ORF YBR159w or A. thaliana ORF AtYBR159. Fatty acid analysis was performed as described for Table 2. The percentage elongation of substrates was calculated using either the formula: Product / (Substrate + Product) * 100, Substrate and Product being the mol. percentage of 20:0 and 22:0 or of 20:1Δ¹¹ and 22:1Δ¹³, respectively; or the formula: (Product1 + Product2) / (Substrate + Product1 + Product2) * 100, Substrate Product1 and Product2 being the mol. percentage of 18:0, 20:0 and 22:0 or of 18:1Δ⁹, 20:1Δ¹¹ and 22:1Δ¹³, respectively.
Figure 1.

18:0 stearic acid
↓ \(\Delta^6\)-desaturase
↓
18:1 oleic acid
↓ \(\Delta^{12}\)-desaturase
↓
18:2 linoleic acid
↓ \(\omega^1\)-desaturase
↓
18:3 \(\alpha\)-linolenic acid
↓ \(\Delta^6\)-desaturase
↓
18:4 octadecatetraenoic acid
↓ \(\omega^3\)-desaturase
↓
20:4 eicosatetraenoic acid

18:2 linoleic acid
↓
18:3 \(\gamma\)-linolenic acid
↓ \(\Delta^6\)-desaturase
↓
20:3 di-\(\gamma\)-linolenic acid
↓ \(\Delta^5\)-desaturase
↓
20:4 arachidonic acid

18:3 \(\gamma\)-linolenic acid
↓ \(\Delta^5\)-desaturase
↓
20:5 eicosapentaenoic acid

\textit{PUFA Elongase}
Figure 2.
Figure 3

WT

+PEA1

18:1vac

+PEA1

20:3

ybr159Δ

+FAE1

+PEA1

20:0

+FAE1

22:0

22:1

detector signal

time (min)
Figure 4A

C4G915 : ~~~~~~~~MDGEVLKNCGCCAGTATSFVKGVTILKFTSFASNNKTFFAKG--KLXVSGAKK--- : 63
YBR159 : ~~~~MTFQQLQEGAGERFRCINGTNAVPGQLKCTTLSRFLAIPLFLFLPA----NFQRYGAP--- : 68
C56G26 : MESSDNLDNHDNLDGNMAGQCPFGQ---AGY----ALAAVA---YRLTVGSIIPIPLDLQRAGGAGG : 70
CG1444 : ~~~~~~~~~~~~~~~~~~~~~~~~~MEEDNSQVLSLSGLAIG---GFCWFR--KVLPGNLEVPGKPFGSVDSLKM--GRVT : 58
F12A21 : ~~~~~~~~~~~~~~~~MEICYTFKSDQYPWFLFLVGSISIFKLELTLSFYYFLRPSKKNLLRLY : 57
Hse17d : ~~~~~~~~~~~~~~~~~~~~~~~~~MDGEVLKNCGCCAGTATSFVKGVTILKFTSFASNNKTFFAKG--KLXVSGAKK--- : 54
Figure 5

Wild-type  Δybr159
+ NADPH  - NADPH  + NADPH  - NADPH

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |

3-Keto

Stearate

3-Hydroxy

origin

Time, minutes
Table 1.

| Gene name/Synonyms | Function / Similarity                                           | Null Mutant Phenotype                        | PUFA elongation |
|--------------------|------------------------------------------------------------------|----------------------------------------------|-----------------|
| YBR159w            | 17-β-hydroxysteroid Dehydrogenase*                               | Lethal/Reduced viability                    | no              |
| YBR265w / TSC10    | 3-ketosphinganine reductase                                      | Lethal in the absence of phytosphinganine   | yes             |
| YMR226c            | Short-chain alcohol dehydrogenase*                              | Viable                                      | yes             |
| YIR036c            | 7-alpha-hydroxysteroid dehydrogenase*                            | Viable                                      | yes             |
| YIR035c            | 11-beta-corticosteroid dehydrogenase*                            | Viable                                      | yes             |
| YIL124w / AYR1     | 1-acyl-dihydroxyacetone phosphate reductase                      | Viable                                      | yes             |
| YDL114w            | Short-chain alcohol dehydrogenase*                              | Viable                                      | yes             |
| YLR426w            | 3-oxoacyl-ACP reductase / alcohol dehydrogenase*                 | Viable                                      | yes             |
| YML131w            | NAD-dependent oxidoreductase*                                    | Viable                                      | yes             |
| YBR046c / ZTA1     | Quinone oxidoreductase                                           | Viable                                      | yes             |
### Table 2.

*mol. % Fatty Acids*

| Episomal gene | BorΔ6 (+LA) | WT | FAT1 (+LA) | WT |
|---------------|-------------|----|-------------|----|
| **Haploid genotype** | **ybr159Δ** | WT | **ybr159Δ** | WT |
| **Fatty acid** | | | | |
| 16:0          | 14.5 ± 0.9  | 15.6 ± 1.2  | 14.1 ± 0.6 | 16.2 ± 0.7 |
| 16:1Δ^9       | 43.5 ± 3.7  | 38.8 ± 2.6  | 48.2 ± 2.4 | 43.8 ± 2.3 |
| 16:2Δ^6,12    | 2.9 ± 1.2   | 3.1 ± 1.1   | –           | –           |
| 18:0          | 4.2 ± 1.0   | 4.2 ± 0.7   | 3.1 ± 0.4   | 4.0 ± 0.3   |
| 18:1Δ^9       | 15.8 ± 1.9  | 13.5 ± 1.3  | 17.1 ± 1.2  | 14.1 ± 0.7  |
| 18:2Δ^6,12 (LA)| 13.9 ± 2.5  | 17.4 ± 2.6  | 16.1 ± 1.5  | 19.7 ± 0.9  |
| α18:3Δ^9,12,15 (ALA) | –         | –         | 1.4 ± 0.5   | 2.2 ± 0.7   |
| γ18:3Δ^6,9,12 (GLA) | 5.2 ± 2.2  | 7.4 ± 1.7  | –           | –           |
| **% desaturation** | | | | |
| 16:1Δ^9       | 6           | 7.5         | –           | –           |
| 18:2Δ^6,12 (LA) | 27        | 29.8        | 8.0         | 10.0        |

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Table 3.

| Fatty acid              | ybr159Δ | WT   | ybr159Δ | WT   | ybr159Δ | WT   |
|-------------------------|---------|------|---------|------|---------|------|
| 16:0                    | 14.2 ± 1.0 | 15.4 ± 3.2 | 11.3 ± 0.6 | 14.3 ± 0.1 | 12.5 ± 1.5 | 16.3 ± 4.4 |
| 16:1Δ9                  | 50.3 ± 5.2 | 39.5 ± 5.1 | 43.9 ± 2.1 | 26.7 ± 0.6 | 49.3 ± 1.0 | 35.6 ± 3.4 |
| 18:0                    | 3.1 ± 0.6 | 3.3 ± 0.8 | 3.7 ± 0.3 | 3.8 ± 0.1 | 2.6 ± 0.2 | 3.5 ± 0.8 |
| 18:1Δ9                  | 27.1 ± 3.5 | 27.8 ± 6.3 | 26.4 ± 4.2 | 21.2 ± 0.1 | 23.8 ± 2.1 | 23.0 ± 4.3 |
| 18:1Δ11 (Vac)           | –       | 6.2 ± 2.5 | 5.8 ± 1.1 | 5.9 ± 0.2 | 6.0 ± 0.4 | 7.5 ± 3.5 |
| γ18:3Δ6,9,12 (GLA)      | 5.3 ± 1.2 | 3.5 ± 2.0 | 5.4 ± 0.5 | 9.8 ± 0.2 | 3.6 ± 0.6 | 7.6 ± 3.0 |
| 20:3Δ8,11,14 (DHGLA)   | –       | 4.3 ± 1.8 | 3.5 ± 0.5 | 17.9 ± 0.5 | 2.2 ± 1.0 | 6.5 ± 2.5 |

% elongation

| Fatty acid              | ybr159Δ | WT | ybr159Δ | WT | ybr159Δ | WT |
|-------------------------|---------|----|---------|----|---------|----|
| 16:1Δ9                  | –       | 13.5 | 11.7 | 18 | 11 | 17.4 |
| γ18:3Δ6,9,12 (GLA)      | –       | 55 | 40 | 65 | 38 | 46 |
Table 4.

| Fatty Acids | Episomal gene 1 | FAE1 |
|-------------|-----------------|------|
| **Episomal gene 2** |  |  |
| **Haploid genotype** | − | + YBR159 | + AtYBR159 |
| ybr159Δ | WT | ybr159Δ | WT | ybr159Δ | WT |

**Fatty acid**

|  | 16:0 | 16:1Δ⁹ | 18:0 | 18:1Δ⁹ | 20:0 | 20:1Δ¹¹ | 22:0 | 22:1Δ¹³ |
|---|------|--------|------|--------|------|--------|------|--------|
| 16:0 | 13.4 ± 0.5 | 8.5 ± 0.9 | 12.2 ± 0.7 | 7.7 ± 0.4 | 11.0 ± 0.7 | 9.6 ± 0.6 |
| 16:1Δ⁹ | 56.9 ± 4.8 | 62.5 ± 1.3 | 69.5 ± 2.1 | 65.3 ± 0.5 | 70.2 ± 2.4 | 63.9 ± 0.8 |
| 18:0 | 3.3 ± 0.2 | 2.5 ± 0.4 | 2.4 ± 0.6 | 2.2 ± 0.1 | 2.4 ± 0.1 | 2.6 ± 0.1 |
| 18:1Δ⁹ | 26.4 ± 4.1 | 15.4 ± 1.3 | 13.0 ± 1.1 | 14.8 ± 0.7 | 14.4 ± 0.9 | 14.7 ± 0.2 |
| 20:0 | − | 2.5 ± 0.5 | 0.7 ± 0.1 | 2.4 ± 0.1 | 0.6 ± 0.1 | 2.0 ± 0.1 |
| 20:1Δ¹¹ | − | 5.7 ± 0.7 | 1.6 ± 0.2 | 5.7 ± 0.1 | 1.1 ± 0.2 | 5.7 ± 0.5 |
| 22:0 | − | 1.4 ± 0.2 | 0.4 ± 0.1 | 1.3 ± 0.2 | 0.2 ± 0.1 | 1.0 ± 0.1 |
| 22:1Δ¹³ | − | 1.1 ± 0.2 | 0.2 ± 0.1 | 0.6 ± 0.1 | 0.1 ± 0.0 | 0.5 ± 0.1 |

**% elongation**

|  | 18:0 | 18:1Δ⁹ | 20:0 | 20:1Δ¹¹ |
|---|------|--------|------|--------|
| 18:0 | − | 60.0 | 31.4 | 62.7 | 25.0 | 53.6 |
| 18:1Δ⁹ | − | 30.6 | 12.2 | 29.9 | 7.7 | 29.7 |
| 20:0 | − | 35.8 | 36.4 | 35.1 | 25.0 | 33.0 |
| 20:1Δ¹¹ | − | 16.2 | 11.1 | 10.0 | 8.3 | 8.0 |
A Saccharomyces cerevisiae gene required for heterologous fatty acid elongase activity encodes a microsomal \( \beta \)-keto-reductase

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*J. Biol. Chem.* published online January 15, 2002

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