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Metabolic engineering of *Saccharomyces cerevisiae* for production of novel lipid compounds

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Abstract The yeast *Saccharomyces cerevisiae* has been modified successfully for production of numerous metabolites and therapeutic proteins through metabolic engineering, but has not been utilized to date for the production of lipid-derived compounds. We developed a lipid metabolic engineering strategy in *S. cerevisiae* based upon culturing techniques that are typically employed for studies of peroxisomal biogenesis; cells were grown in media containing fatty acids as a sole carbon source, which promotes peroxisomal proliferation and induction of enzymes associated with fatty acid β -oxidation. Our results indicate that growth of yeast on fatty acids such as oleate results in extensive uptake of these fatty acids from the media and a subsequent increase in total cellular lipid content from 2% to 15% dry cell weight. We also show that co-expression of plant fatty acid desaturases 2 and 3 (*FAD2* and *FAD3*), using a fatty acid-inducible peroxisomal gene promoter, coupled the processes of fatty acid uptake with the induction of a new metabolic pathway leading from oleic acid (18:1) to linolenic acid (18:3). Finally, we show that cultivation of yeast cells in the presence of triacylglycerols and exogenously supplied lipase promotes extensive incorporation of triglyceride fatty acids into yeast cells. Collectively, these results provide a framework for bioconversion of low-cost oils into value-added lipid products.

Introduction

Metabolic engineering is an emerging field of biotechnology which offers tremendous potential for the pro-

duction of desired metabolites. The experimental approach is based upon the manipulation of endogenous genes or the introduction of foreign genes into an organism of interest in order to reroute metabolic pathways for the production of specific compounds (Stephanopoulos 1999). The successful development of an engineering process is dependent upon a thorough working knowledge of the genes and metabolites in question and the availability of a robust set of tools for analyzing physiological changes within the host organism. The yeast *Saccharomyces cerevisiae* has served as a model organism for the development of metabolic engineering strategies geared towards the production of certain metabolites and therapeutic proteins (Ostergaard et al. 2000). However, little attention has been paid to the development of lipid-based technologies, largely because of the low lipid content of *S. cerevisiae* (Jacob 1993).

Our previous work has focused on understanding the molecular mechanisms involved in peroxisomal biogenesis, with emphasis on the targeting and assembly of peroxisomal membrane proteins (Dyer et al. 1996; Mullen and Trelease 2000). For these studies, both plants and *S. cerevisiae* were used as model systems. In *S. cerevisiae*, fatty acids are degraded exclusively within peroxisomes, and the identification of yeast mutants that no longer grow on fatty acids as a sole carbon source has permitted the identification of many genes involved in peroxisomal biogenesis (the *PEX* genes) (Subramani et al. 2000). During our studies with *S. cerevisiae*, we observed that cells cultivated on free fatty acids such as oleic acid contained large amounts of lipid droplets not observed in cells growing on simpler carbon sources such as glucose. The current study was initiated to characterize the lipid content of *S. cerevisiae* further, and determine whether certain lipid-modifying enzymes might be expressed under the control of a *PEX* gene promoter to modify fatty acid composition and produce new lipid products.

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Materials and methods

Yeast strains and culturing conditions

Saccharomyces cerevisiae strains MMYO11 α (*MAT α ade 2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Ole⁺*), MM Δ PX5 (*MAT α pex5::HIS3 ade2::Ppgk-GFPAKL-URA3 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Ole⁺*), or derivatives thereof (see below) were used throughout these studies. Untransformed yeast cells were maintained on YPD media (1% yeast extract, 2% peptone, 2% dextrose) solidified with 2% agar. Cells containing plasmids were maintained on SD plates (2% dextrose, 0.67% yeast nitrogen base without amino acids, 2% agar) with appropriate amino acid supplements. Single colonies were inoculated into SD liquid media and grown overnight at 30°C and 300 rpm. Cells were diluted into a variety of media (see below) and grown at 20°C and 300 rpm in a Forma Scientific Model 4580 refrigerated console incubator/shaker. Cultivation at 20°C was chosen since optimal activity of the *Brassica napus* fatty acid desaturase 3 (*FAD3*) enzyme expressed in yeast was previously observed at this temperature (Dyer et al. 2001). Cells were diluted to 0.1 OD₆₀₀ in SD media and grown for approximately 20 h, diluted to 0.25 OD₆₀₀ in SGal media (2% galactose, 0.67% yeast nitrogen base) and grown for approximately 40 h, or diluted to 1.0 OD₆₀₀ in SGd (3% glycerol, 0.1% dextrose, 0.67% yeast nitrogen base) for 40 h. Cells grown in oleic acid media (composition below) were pre-cultured in SGd overnight rather than SD. The following day, cultures were “boosted” by adding 0.1 volume of 10% yeast extract and 20% peptone then cultured for 4 h. Cells were harvested by centrifugation and re-inoculated at 1.0 OD₆₀₀ in mineral medium (van Dijken et al. 1976) containing 0.1% v/v oleic, linoleic, or linolenic acids and appropriate amino acid supplements, and cultured for 40 h. Cells were cultured for different lengths of time to accommodate differences in growth rate on the various carbon sources. All cultures were harvested at late-log/stationary phase. In some experiments, free fatty acids were replaced by 0.1% v/v trlinolein and incubated with or without detergent (1% v/v Tween 40 – polyoxyethylenesorbitan monopalmitate) and lipase (1 mg/ml lipase, type VII isolated from *Candida rugosa*; Sigma).

Determination of dry cell weight and extraction of yeast lipids

Cells obtained from a 100 ml culture were divided into two 45 ml portions, one for determination of yeast dry cell weight and the other for extraction of yeast lipids. To determine dry cell weight, cells were collected by centrifugation, washed twice with water, then frozen at –80°C and lyophilized using a FTS Systems Flexi-Dry MP lyophilizer. For extraction of yeast lipids, cells in the other 45 ml portion were converted to spheroplasts by enzymatic digestion of cell walls, as previously described (Dyer et al. 1996), then lipids were extracted using the chloroform–methanol procedure of Bligh and Dyer (1959). All organic solvents contained 0.01% butylated hydroxytoluene as an antioxidant. Total lipid content of yeast cells was calculated as the percentage of dry cell weight.

Thin-layer and gas chromatography

Lipid classes were separated by thin-layer chromatography using Silica gel 60 HPTLC plates (10×10 cm) and a mobile phase consisting of hexane:diethyl ether:glacial acetic acid (80/20/1 v/v/v). Lipid components were visualized by spraying with 3.3% cupric acetate in 8% phosphoric acid and charring at 140°C for 10 min on a VPF visualization chamber (Analtech), then identified by comparison with R_f of lipid standards. Fatty acid methyl esters (FAME) were prepared from yeast total lipid extracts using sodium methoxide, which derivatizes fatty acyl-glycerolipids including neutral lipids and phospholipids, but not free fatty acids. Where indicated, FAME were prepared using 1 M methanolic-HCl (Alltech) to ensure that cellular free fatty acids were included in

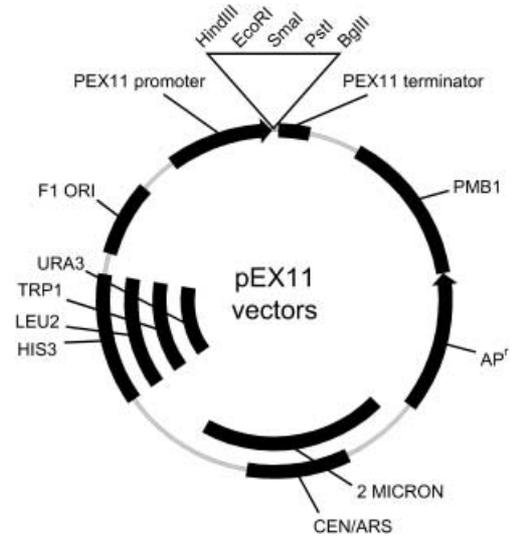


Fig. 1 Vectors for fatty acid-inducible gene expression in yeast. An expression cassette based on the yeast fatty acid-inducible *PEX11* promoter was constructed in a series of low- and high-copy yeast shuttle vectors containing four different selectable markers (Sikorski and Hieter 1989; Christianson et al. 1992), resulting in eight different expression plasmids

the compositional analysis. FAME were extracted with hexane, passed over sodium sulfate, and hexane volume was reduced under a gentle stream of nitrogen. FAME were analyzed on a Hewlett Packard 5890 Series II Gas Chromatograph equipped with an autoinjector and splitless injection, flame ionization detector, and a 30 m 0.53 mm Supelco SP-2380 column, using helium gas as a carrier. A temperature program of 110–160°C at 15°C/min, to 170°C at 5°C, then to 200°C at 22.5°C/min with a final hold for 3.3 min was employed. FAME were identified by comparison of retention times to standards, using methyl heptadecanoate as an internal standard, and % FAME was calculated based on peak area counts.

Vector construction and yeast strains

A series of yeast expression vectors was constructed by transferring a DNA fragment containing the yeast *PEX11* gene promoter and terminator, separated by a short polylinker of restriction sites, to the pRS series of yeast shuttle vectors (Sikorski and Hieter 1989; Christianson et al. 1992). Each *PEX11*-based shuttle vector contains one of four selectable yeast markers (*HIS3*, *TRP1*, *LEU2*, *URA3*), and is available in both low- and high-copy number versions (Fig. 1). The *PEX11* promoter and terminator fragment was obtained from plasmid pKS-27poly (Dyer et al. 1996), which contains the *PEX11* promoter, a polylinker (5′-*Hind*III, *Eco*RI, *Sma*I, *Pst*I, and *Bgl*II-3′), and terminator cloned into the *Clal/Bam*HI sites of pBluescript KS- (Stratagene). The restriction sites within the polylinker between the *PEX11* promoter and terminator are not unique within all the pRS vectors (Sikorski and Hieter 1989; Christianson et al. 1992). The *PEX11* expression cassette was excised from pKS-27poly using *Kpn*I/*Sac*I, gel purified, and ligated into similarly digested yeast shuttle vectors pRS-314, pRS-424, pRS-316, and pRS-426 to generate plasmids pEX11-314 (*CEN6 ARSH4*, *TRP1*), pEX11-424 (2 μ , *TRP1*), pEX11-316 (*CEN6 ARSH4*, *URA3*), and pEX11-426 (2 μ , *URA3*), respectively. The cassette was transferred to pRS-313 and pRS-315 using *Xho*I/*Xba*I to generate pEX11-313 (*CEN6 ARSH4*, *HIS3*) and pEX11-315 (*CEN6 ARSH4*, *LEU2*), respectively. Finally, the cassette was transferred to pRS-423 and pRS-425 using *Xho*I/*Sac*I to generate pEX11-423 (2 μ , *HIS3*) and pEX11-425 (2 μ , *LEU2*), respectively.

Yeast expression vectors containing *PEX11*-driven plant fatty acid desaturases (FADs) were constructed using myc-epitope tagged *Arabidopsis thaliana* FAD2 and hemagglutinin (HA)-tagged *Brassica napus* FAD3, which were previously used to characterize desaturase subcellular localization (Dyer and Mullen 2001) and post-transcriptional regulation (Dyer et al. 2001). The myc-tagged FAD2 ORF was ligated between the yeast *PEX11* promoter and terminator by transferring an *EcoRI/PstI* fragment from pKS-myc-FAD2 (Dyer and Mullen 2001) to similarly digested pEX11-424. The final plasmid was called pEX11-424-mycFAD2 (2μ , *TRP1*). The HA-tagged FAD3 ORF was cloned between the *PEX11* promoter and terminator by transferring an *EcoRI/PstI* fragment from plasmid pKS-HAB1 (Dyer et al. 2001) to the *EcoRI/PstI* sites of pKS-27poly. The resulting plasmid was called pKS-27HAB1. The gene cassette was transferred from this plasmid as a *SalI/SacI* fragment to the *SalI/SacI* sites of pRS-425 to generate plasmid pEX11-425-HAFAD3 (2μ , *LEU2*). Plasmids were transfected singly or in combination into MMYO11 α or MMAPX5 yeast cells using the lithium acetate method of Gietz and Woods (1994). Yeast strains were deposited into the Agricultural Research Service Culture Collection (National Center for Agricultural Utilization Research, 1815 N. University St., Peoria, IL 61604, USA) and are available upon request. The strain names, descriptions, and accession numbers are: MM-424, *S. cerevisiae* strain MMYO11 α harboring control vector pRS-424 (2μ , *TRP1*), NRRL Y-27443; MM-F2, MMYO11 α harboring plasmid pEX11-424-mycFAD2, NRRL Y-27444; MM-F3, MMYO11 α harboring plasmid pEX11-425-HAFAD3, NRRL Y-27445; MM-F2/F3, MMYO11 α harboring plasmids pEX11-424-mycFAD2 and pEX11-425-HAFAD3, NRRL Y-27446; MMAPX5-424, *S. cerevisiae* strain MMAPX5 harboring control plasmid pRS-424, NRRL Y-27447; MMAPX5-F2/F3, MMAPX5 harboring plasmids pEX11-424-mycFAD2 and pEX11-425-HAFAD3, NRRL Y-27448.

Preparation of protein extracts, SDS-PAGE, and Western blotting

Total protein extracts were obtained from yeast using the method of Veenhuis and Goodman (1990) with slight modification. Briefly, cells were incubated in 5% trichloroacetic acid prior to glass bead disruption. Lysates were not boiled, however, to improve recovery of plant fatty acid desaturase proteins (personal observation). Protein concentration was determined on triplicate samples using the BioRad protein assay with BSA as a standard. Fifteen micrograms of protein from each whole cell lysate were separated on 10% SDS-polyacrylamide gels and either stained with Coomassie R-250 or transferred to nitrocellulose for Western blotting. Enhanced chemiluminescence detection was performed using substrate and Hyperfilm from Amersham-Pharmacia, as described by the manufacturer. Antibodies used in these studies included the anti-HA mouse mAb 12CA5 (1 μ g/ml) (Boehringer Mannheim-Roche), anti-c-myc mouse mAb 9E10 (1:1,000) (BabCo), anti-dolichol phosphate mannose synthase mouse mAb 5C5-A7 (4 μ g/ml) (Molecular Probes), and goat-anti-mouse IgG horseradish peroxidase conjugate (1:10,000 dilution) (Biosource International).

Results

Uptake of fatty acids by *S. cerevisiae*

S. cerevisiae cells were grown in the presence of multiple carbon sources, or fatty acids alone, to determine the effects of media composition on uptake and assimilation of fatty acids from the growth media. Yeast cells cultured on galactose contained low amounts of total cellular lipids, which were distributed primarily in phospholipid and neutral lipid fractions (Fig. 2). Addition of ole-

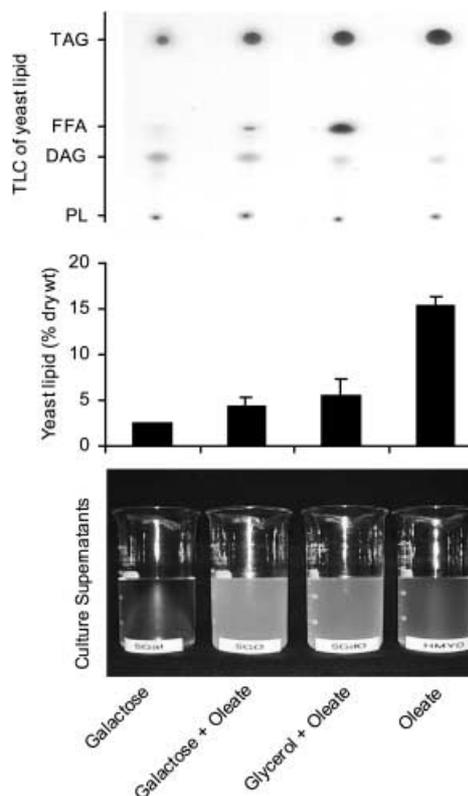


Fig. 2 Uptake of fatty acids from the culture media by *Saccharomyces cerevisiae* and subsequent increase in total yeast lipid content. Yeast strain MM-424 was grown in liquid media containing either galactose, galactose and oleate, glycerol and oleate, or oleate alone. Oleic acid was included in the media at 0.1% (v/v), when added. The *bottom panel* shows supernatants obtained after centrifugation of the media to remove yeast cells. The *middle panel* shows the total lipid content of yeast cells cultured in the respective media. The *top panel* shows a representative TLC separation of 6 μ g of lipid obtained from each yeast lipid extract. Positions of lipid standards are shown to the *left*: TAG triacylglycerol, FFA free fatty acid, DAG diacylglycerol, PL phospholipid. All experiments were carried out at least three times

ic acid to the galactose media led to a small increase in total cellular lipid content and an increase in triacylglycerol and free fatty acid content was observed. The increase in cellular fatty acid content was more pronounced when cells were grown on a mixture of glycerol and oleic acid (Fig. 2). Cultivation of *S. cerevisiae* in media containing oleic acid as a sole carbon source, however, resulted in more extensive uptake of oleate from the media, with a sevenfold increase in total cellular lipid content from approximately 2% to 15% dry cell weight. TLC separation of lipids obtained from oleic acid-grown cells revealed that the majority of fatty acids were esterified to triacylglycerols. Furthermore, the amount of cellular free fatty acids was substantially reduced compared with other carbon sources, particularly mixtures of galactose/oleate and glycerol/oleate. Cultivation of *S. cerevisiae* in media containing linoleic or linolenic acids as sole carbon sources gave similar results to those cultured on oleic acid (results not shown).

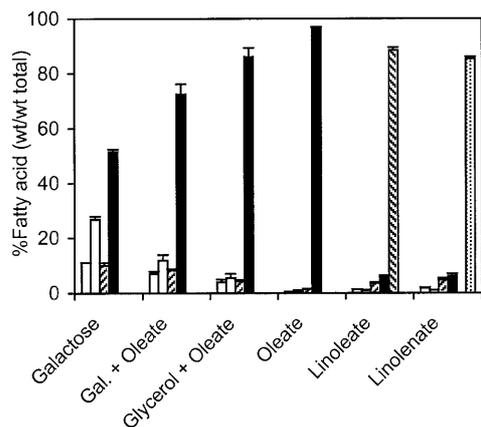


Fig. 3 Fatty acid composition of *S. cerevisiae* cells cultured in the presence of various carbon sources. *S. cerevisiae* cells (strain MM-424) were grown in media containing the indicated carbon sources, then yeast cells were harvested and fatty acid composition determined using GC. FAME were prepared using methanolic-HCL to ensure that cellular free fatty acids were included in the compositional analysis. Values reported represent the average and standard deviation of four independent experiments. Bars represent palmitic (empty), palmitoleic (shaded), stearic (diagonal stripes up), oleic (solid), linoleic (diagonal stripes down), linolenic (spots)

S. cerevisiae cells cultured in galactose media exhibit a simple fatty acid composition dominated by palmitic (16:0), palmitoleic (16:1), stearic (18:0), and oleic (18:1) acids (Fig. 3). Inclusion of oleic acid in the galactose or glycerol media resulted in an increase in cellular oleic acid (18:1) content and reduction of palmitoleic acid (16:1). Growth on oleic acid as a sole carbon source resulted in even greater incorporation of oleic acid, accounting for approximately 95% of total yeast fatty acids (Fig. 3). Cultivation of *S. cerevisiae* in the presence of linoleic (18:2) or linolenic (18:3) acids resulted in similar uptake and incorporation of the exogenously supplied fatty acid, accounting for 85–90% of fatty acid composition (Fig. 3). The uptake of these unsaturated fatty acids from the growth media led to a severe reduction in the amount of endogenously synthesized mono-unsaturated fatty acids (16:1 and 18:1), which was likely due to repression of endogenous yeast desaturase activity (Trotter 2001).

To determine whether *S. cerevisiae* could also utilize triacylglycerol lipids, which are major components of common vegetable oils, yeast cells were cultured in the presence of trilinolein, then cells were harvested and fatty acid composition determined using GC (Fig. 4). *S. cerevisiae* cells do not typically contain linoleic acid, and the appearance of this fatty acid in yeast lipids can be used to gauge fatty acid uptake. Detergent was also included in these studies to help solubilize triacylglycerols. Cells cultivated in the presence of free linoleic acid as a sole carbon source contained over 80% linoleic acid, whereas cells cultivated in the presence of trilinolein contained only 1% linoleic acid (Fig. 4). Inclusion of a nonspecific lipase in the media containing trilino-

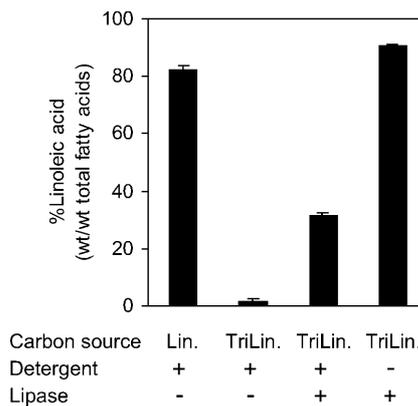


Fig. 4 Influence of a nonspecific lipase on uptake of fatty acids in growth media containing triglycerides. Yeast cells (strain MM-424) were cultured in the presence of free linoleic acid (*Lin.*), trilinolein (*TriLin.*), detergent (1% v/v polyoxyethylenesorbitan monopalmitate – Tween 40), or lipase (1 mg/ml), then lipids were extracted and fatty acid composition determined using GC. Content of linoleic acid in yeast lipids is shown. Values represent the average and standard deviation of three independent experiments

lein, however, facilitated uptake of linoleic acid, accounting for 30% of total fatty acids. Analysis of total fatty acid composition of these cells revealed that palmitic acid content was abnormally high, accounting for over 40% of total fatty acids (data not shown). This suggested that the lipase preferentially hydrolyzed the palmitoyl moiety off of the detergent, which was added in tenfold excess over the triacylglycerol in the growth media. Incubation of yeast cells in the presence of trilinolein and lipase, without detergent, resulted in an uptake of linoleic acid comparable to the amounts observed when linoleic acid was provided as a free fatty acid in the media (Fig. 4). Thus, the inclusion of a detergent is neither necessary nor desirable for hydrolysis and uptake of triglyceride fatty acids.

Expression of lipid-modifying enzymes in yeast using a fatty acid-inducible gene promoter

To couple the processes of fatty acid uptake into *S. cerevisiae* cells and expression of lipid-modifying enzymes, plant fatty acid desaturases 2 and 3 (*FAD2*, *FAD3*) were expressed in yeast cells using the fatty acid-inducible yeast *PEX11* gene promoter. The *PEX11* gene encodes a protein (Pex11p) that is involved in proliferation of peroxisomes in response to certain environmental cues (Erdmann and Blobel 1995; Marshall et al. 1995). The *PEX11* gene is expressed at low basal levels in glucose and induced approximately 100-fold when cells are shifted to media containing fatty acids as a sole carbon source (Marshall et al. 1995). The plant *FAD2* enzyme converts oleic acid (18:1) to linoleic acid (18:2), and *FAD3* converts linoleic acid (18:2) to linolenic acid (18:3) (Covello and Reed 1996; Reed et al. 2000). Both enzymes are membrane-bound proteins located in the en-

Table 1 Lipid metabolic engineering in *Saccharomyces cerevisiae*

| Yeast strain | Enzymes ^a | Carbon source | %FAME (wt/wt total) ^b | | |
|--------------|----------------------|---------------|----------------------------------|-----------|------------|
| | | | Oleate | Linoleate | Linolenate |
| MM-424 | | Dextrose | 53.8±1.4 | – | – |
| MM-F2/F3 | FAD2 + FAD3 | Dextrose | 32.6±2.8 | 17.6±1.8 | 1.07±0.32 |
| MM-424 | | Oleic acid | 97.6±0.4 | – | – |
| MM-F2/F3 | FAD2 + FAD3 | Oleic acid | 82.8±2.3 | 11.9±1.8 | 0.34±0.13 |
| MMΔPX5-424 | | Dextrose | 54.8±0.6 | – | – |
| MMΔPX5-F2/F3 | FAD2 + FAD3 | Dextrose | 27.4±2.6 | 21.8±1.4 | 1.26±0.10 |

^a Plant *FAD2* and *FAD3* desaturases were ectopically-expressed on high-copy plasmids. Gene expression was driven by the yeast *PEX11* gene promoter

^b Percentages of 18 carbon, unsaturated fatty acids are shown. Values represent the average and standard deviation of three independent experiments

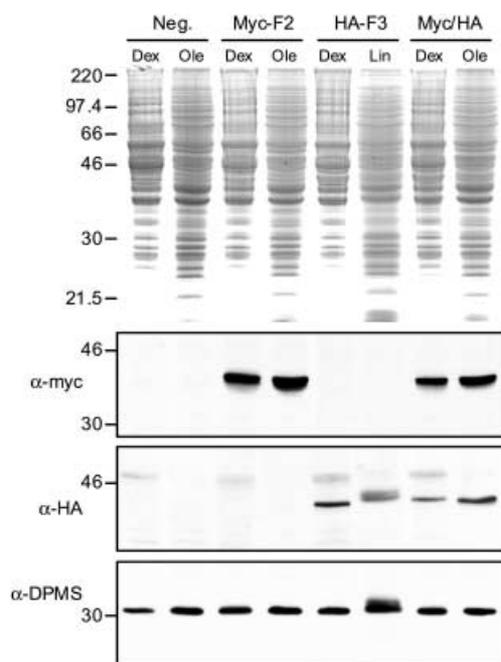


Fig. 5 SDS-PAGE and Western blot analysis of plant *FAD2* and *FAD3* expressed in yeast cells using the *PEX11* gene promoter. Individual yeast strains, designated at the top of the figure, are: Neg. control strain MM-424, *Myc-F2* strain MM-F2 (contains myc-epitope tagged *FAD2*), *HA-F3* strain MM-F3 (contains HA-tagged *FAD3*), *Myc/HA* strain MM-F2/F3 (contains both myc-tagged *FAD2* and HA-tagged *FAD3*). Cells were cultured in media containing the indicated carbon source (listed at the top of each lane), then total proteins were extracted, separated by SDS-PAGE, and either stained with Coomassie blue (upper panel) or transferred to nitrocellulose for Western blotting using the indicated antibodies (bottom panels). Positions of molecular mass markers (kDa) are shown to the left. *Dex* dextrose, *Ole* oleic acid, *Lin* linoleic acid, α -myc anti-myc antibody, α -HA anti-HA antibody, α -DPMS anti-dolichol phosphate mannosyl synthase antibody, a marker protein for the yeast endoplasmic reticulum

doplasmic reticulum (Dyer and Mullen 2001). The desaturases were epitope-tagged and expressed singly or in combination in *S. cerevisiae* cells using the *PEX11* promoter, then protein levels were evaluated by Western blot analyses. The myc-Fad2 protein was detected in lysates obtained from cells cultured in both dextrose and oleic acid medium (Fig. 5). Similar results were ob-

served for HA-Fad3 obtained from cells grown in dextrose and linoleic acid (the physiological substrate for *FAD3*). Co-expression of myc-Fad2 and HA-Fad3 resulted in the appearance of both proteins within yeast cells (Fig. 5). Expression of *FAD2* and/or *FAD3* did not influence the steady state amount of dolichol phosphate mannosyl synthase, a marker protein for the yeast endoplasmic reticulum membrane (Horazdovsky and Emr 1993).

Lipid metabolic engineering in *S. cerevisiae*

S. cerevisiae cells harboring *PEX11*-driven *FAD2* and *FAD3* high-copy plasmids exhibited changes in fatty acid composition consistent with the establishment of a new metabolic pathway leading from oleic acid (18:1) to linolenic acid (18:3), via linoleic acid (18:2) (Table 1). Control yeast cells cultured on dextrose contained approximately 54% oleic acid, while yeast cells expressing plant *FAD2* and *FAD3* genes showed a reduction in oleic acid content and appearance of linoleic and linolenic acids, which accounted for approximately 18% and 1.1% of total fatty acids, respectively (Table 1). Growth of control cells on media containing oleic acid as a sole carbon source resulted in substantial incorporation of oleic acid into yeast lipids, accounting for 98% of total cellular fatty acids (Table 1). Co-expression of *FAD2* and *FAD3* led to conversion of a portion of the oleic acid into linoleic and linolenic acids, accounting for 12% and 0.3% of total fatty acids, respectively (Table 1).

The *FAD2* and *FAD3* cDNAs were also expressed in *S. cerevisiae* cells containing a disruption of the *PEX5* gene (strain MMΔPX5). These mutant cells, disrupted in peroxisomal biogenesis, are unable to grow on fatty acids as a sole carbon source, but are able to grow on fermentable carbon sources such as dextrose (Subramani et al. 2000). The fatty acid composition of control strain MMΔPX5-424, cultivated in dextrose, was similar to the composition of the wild-type control strain, MM-424, with oleic acid accounting for approximately 55% of total fatty acid composition (Table 1). Co-expression of plant *FAD2* and *FAD3* desaturases in MMΔPX5 cells led to the conversion of a portion of the oleic acid into lino-

leic and linolenic acids, accounting for approximately 22% and 1.3% of total fatty acids, respectively (Table 1).

Discussion

Here we describe a lipid metabolic engineering strategy in *S. cerevisiae* that couples the processes of fatty acid uptake from the growth media with expression of fatty acid-modifying enzymes. A key component in this process was cultivation of yeast cells in media containing fatty acids as a sole carbon source, which enhanced the uptake of free fatty acids and resulted in a sevenfold increase in the total cellular lipid content. Although the growth of *S. cerevisiae* on fatty acids as a sole carbon source induces the proliferation of peroxisomes and associated enzymes for fatty acid β -oxidation, yeast cells grow very poorly on this carbon source, with a doubling time of approximately 20 h. This provides ample time for the modification of fatty acid composition by transgenically-expressed enzymes.

Significant insight into the physiological changes associated with the growth of *S. cerevisiae* on fatty acids can be obtained from the studies of Kal et al. (1999), who previously performed a genome-wide analysis of changes in gene expression when cells were cultured on glucose or oleic acid as a sole carbon source. Their results revealed that cultivation of cells on oleic acid induced the expression of many genes involved in fatty acid metabolism, including metabolite transporters and enzymes associated with fatty acid degradation. Among the induced genes were the fatty acyl-CoA synthetases, which have been implicated in the acquisition of fatty acids from the growth media (Fargeman et al. 2001). These enzymes catalyze the formation of fatty acyl-CoAs from free fatty acids and CoA, thereby allowing fatty acids obtained from the media to enter the cellular pool of acyl-CoAs. The fatty acyl-CoAs are readily incorporated into both neutral lipids and phospholipids (Walenga and Lands 1975), which could explain the increase in total cellular lipid content observed here (Fig. 2). Induction of fatty acyl-CoA synthetase activity may also be responsible for the reduction of free fatty acid content in cells cultured on oleic acid as a sole carbon source (Fig. 2).

We expressed two plant fatty acid desaturases in *S. cerevisiae* using the *PEX11* gene promoter, since this gene is expressed at low but detectable levels in dextrose and induced 100-fold when cells are grown on oleic acid as a sole carbon source (Marshall et al. 1995; Kal et al. 1999). Surprisingly, investigation of steady-state levels of desaturase proteins by Western blotting revealed that the amounts of plant desaturase proteins did not increase appreciably when cells were shifted from dextrose to oleic acid media (Fig. 5). Although the reason for this lack of inducement is currently unknown, it might reflect differences in *FAD* mRNA stability, translational efficiency, or rates of protein turnover between cells cultured on dextrose versus oleic acid. Nevertheless, ex-

pression of the desaturases in dextrose- or oleate-grown cells was sufficient for remodeling fatty acid composition, consistent with the introduction of a new metabolic pathway leading from oleic acid (18:1) to linolenic acid (18:3) (Table 1).

We also expressed the plant fatty acid desaturases in a strain of *S. cerevisiae* disrupted in the *PEX5* gene. The *PEX5* gene encodes the receptor for the majority of proteins destined for the peroxisomal matrix, and cells lacking this gene are unable to grow on fatty acids as sole carbon source due to their inability to degrade fatty acids (Subramani et al. 2000). The rationale for performing our experiments in a *PEX5*-disrupted yeast strain was that, in certain lipid metabolic engineering strategies, it can be envisioned that desired fatty acid end products might be selectively hydrolyzed from membranes and targeted for degradation within peroxisomes (Eccleston and Ohlrogge 1998; Millar et al. 2000). In such cases, lipid engineering might be performed in yeast strains disrupted in their ability to degrade fatty acids altogether. Results presented in Table 1 indicate that lipid metabolic engineering experiments can be performed in *PEX5*-disrupted *S. cerevisiae* cells that are cultivated on dextrose as a carbon source, with fatty acid products accumulating to levels comparable to those observed in wild-type cells expressing the plant enzymes. However, the inability of the *PEX5*-disrupted yeast cells to survive on fatty acids as a sole carbon source would limit their utilization to lipid engineering strategies focused on conversion of endogenous yeast lipids, rather than exogenously supplied lipids, into new lipid products. Alternatively, *PEX5*-disrupted yeast cells might be further engineered to circumvent this limitation. For example, constitutive, high-level expression of fatty acyl-CoA ligases might permit robust uptake and assimilation of fatty acids from the growth media, even when an additional carbon source, such as dextrose, is included in the media.

Finally, one of our long-term goals is to develop a microbial expression system for bioconversion of low-cost vegetable oils into value-added products. Vegetable oils are composed primarily of triacylglycerols, and *S. cerevisiae* is typically unable to grow on these compounds as a carbon source. Here, we demonstrate that inclusion of a nonspecific lipase in media containing triacylglycerols facilitates hydrolysis and subsequent uptake of fatty acids into *S. cerevisiae* cells (Fig. 4). This suggests that *S. cerevisiae* might be engineered to secrete large amounts of lipase for breakdown of triglycerides and incorporation of fatty acid components. Such strains of *S. cerevisiae* have previously been described (Okkels 1996). A combination of the culturing methods described here, and expression of genes for both incorporation of fatty acids and their modification, may permit the industrial-scale utilization of *S. cerevisiae* for bioconversion of low-cost starting materials into value-added lipid products.

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