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Lipidome and proteome of lipid droplets from the methylotrophic yeast *Pichia pastoris*

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ABSTRACT

Lipid droplets (LD) are the main depot of non-polar lipids in all eukaryotic cells. In the present study we describe isolation and characterization of LD from the industrial yeast *Pichia pastoris*. We designed and adapted an isolation procedure which allowed us to obtain this subcellular fraction at high purity as judged by quality control using appropriate marker proteins. Components of *P. pastoris* LD were characterized by conventional biochemical methods of lipid and protein analysis, but also by a lipidome and proteome approach. Our results show several distinct features of LD from *P. pastoris* especially in comparison to *Saccharomyces cerevisiae*. *P. pastoris* LD are characterized by their high preponderance of triacylglycerols over steryl esters in the core of the organelle, the high degree of fatty acid (poly)unsaturation and the high amount of ergosterol precursors. The high phosphatidylinositol to phosphatidylserine of ~7.5 ratio on the surface membrane of LD is noteworthy. Proteome analysis revealed equipment of the organelle with a small but typical set of proteins which includes enzymes of sterol biosynthesis, fatty acid activation, phosphatidic acid synthesis and non-polar lipid hydrolysis. These results are the basis for a better understanding of *P. pastoris* lipid metabolism and lipid storage and may be helpful for manipulating cell biological and/or biotechnological processes in this yeast.

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1. Introduction

The methylotrophic yeast *Pichia pastoris* is widely used for heterologous protein expression [1–4]. Despite its extensive commercial use the cell biological characterization of this yeast is lacking behind. For this reason our laboratory initiated a systematic approach to investigate *P. pastoris* organelles with the emphasis on the characterization of biomembranes and lipids [5,6]. Despite the progress which we made a number of subcellular compartments remained uncharacterized so far. As an example, isolation and characterization of lipid droplets (LD) from *P. pastoris* have not yet been reported. LD, also named lipid particles or oil bodies are specific subcellular compartments which gained much interest recently regarding their emerging role in health and

1388-1981 © 2012 Elsevier B.V. Open access under CC BY-NC-ND license. http://dx.doi.org/10.1016/j.bbalip.2012.09.017 disease [7]. They mainly function as depots of excess lipids (sterols and fatty acids) in the biological inert form of triacylglycerols (TG) and steryl esters (SE), but also contribute to non-polar lipid synthesis and mobilization [8]. LD are supposed to originate from the ER by a budding process, although steps and mechanism(s) leading to the biogenesis of this organelle are still a matter of dispute. Alternative models for LD formation have also been proposed (for reviews, see Refs. [9–14]). Recent studies in *Saccharomyces cerevisiae* advocated structural and functional connection between ER and LD and proposed the possibility of protein exchange between these two compartments [15].

The general structure of LD is similar in all eukaryotic cells (for reviews, see [8,9]). Yeast LD consist of a hydrophobic core formed by TG and SE encompassed by a phospholipid monolayer with a small number of proteins embedded [16,17]. Most recently, more than 90 proteins were allocated to LD from *S. cerevisiae* [18]. Many of these polypeptides participate in lipid metabolism, such as phosphatidate and sterol synthesis [19,20], fatty acid activation [21–23], and TG and SE synthesis/lipolysis [24–30]. Besides lipid metabolic functions several other functions unrelated to lipid metabolism were assigned to LD, such as storage and sequestration of protein aggregates and incorrectly folded proteins [9,31].

Abbreviations: TG, triacylglycerols; SE, steryl esters; LD, lipid droplets; PA, phosphatidic acid; LP, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPE, dimethyl-PE; CL, cardiolipin; DMCD, 4,14-dimethyl-cholesta-8,24-dienol; MS, mass spectrometry; GFP, green fluorescent protein; WT, wild type

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TG and SE comprise the highly hydrophobic core of LD. In S. cerevisiae, these two major non-polar lipids are synthesized by four enzymes [32]. TG are formed via acylation of diacylglycerols (DAG) with the fatty acid moiety derived from different sources. The diacylglycerol:phospholipid acyltransferase Lro1p possesses phospholipase A₂ (B) and acyltransferase activities and catalyzes TG formation in an acyl-CoA-independent manner utilizing phospholipids, especially phosphatidylethanolamine as acyl donor [33,34]. The second TG synthesizing enzyme, Dga1p, esterifies DAG in an acyl-CoA-dependent way and requires activated fatty acids as co-substrates. Lro1p is found exclusively in the ER whereas Dga1p is dually localized to LD and ER [35]. Are1p and Are2p from S. cerevisiae are two homologous SE synthases [36,37]. They are mainly present in the ER and esterify sterols with fatty acids using acyl-CoA as fatty acid donor [20]. Are2p has the major acyl CoA:sterol acyltransferase activity in S. cerevisiae and predominantly forms esters of ergosterol, the final product of the sterol biosynthetic pathway in yeast. Are1p esterifies ergosterol precursors as well as ergosterol and has elevated activity under hypoxic conditions [36,38–40]. Noteworthy, only one acyl CoA:sterol acyltransferase, Are2p, has been annotated in the *P. pastoris* genome database [41].

In the present report we extend our knowledge about LD biochemistry and cell biology to *P. pastoris* and compare these data to the wellestablished model yeast *S. cerevisiae*. The strategy to characterize LD from *P. pastoris* cells included (i) isolation of highly pure organelles; (ii) conventional biochemical analysis of lipid components; and (iii) mass spectrometric (MS) analysis of lipids and proteins. Especially the lipidome and proteome studies allowed us to investigate LD from *P. pastoris* at the molecular level which may become highly relevant for biotechnological applications.

2. Experimental procedures

2.1. Strains and culture conditions

P. pastoris X33 (MATa, Mut⁺, His⁻) and *P. pastoris* X33_*GFP-ERG6* (MATa, Mut⁺, His⁺) strains were used throughout this study. Cells were grown under aerobic conditions to the early stationary phase (26 h) at 30 °C in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid) and 2% glucose (Merck). Media were inoculated to a starting OD₆₀₀ of 0.1 from precultures grown aerobically for 48 h in YPD medium at 30 °C.

2.2. Construction of GFP-PpErg6p expression vector

The primer pair (GFP-fwd CGCGGATCCGCGTTTTTGTAGAAATGT CTTG GTGTCCTCGTCCAATCAGGTAGCCATCTCTG and GFP-rev ATAG TTTAGCGGCC GCCTCGAGCCCGGGATTTAAATACTTGTACAATTCATCCA TGCCATGTGTAATCCCAGCAGCAGT) was used for amplifying a GAP promoter fused to a Cy3-GFP open reading frame lacking the stop codon. The PCR product was inserted into BamHI and NotI restriction sites at the multiple cloning site of the pPIC3.5 plasmid. Primer GFPrev also contained additional recognition sites enabling N-terminal fusion of GFP to genes of interest. The *PpERG6* open reading frame was amplified from genomic DNA using the forward primer ERG6fwd (5'-GCGCGATTTAAATATGACTACCT CTACAACTGAACAAG-3') which contained a Smil site and the reverse primer ERG6-rev (5'-CAATGCG GCCGCTTATTTGGCATCCAATGGTTTTC-3') with a NotI site. PpERG6 was inserted within the corresponding sites of the aforementioned vector behind the GFP gene. The quality of the final construct was confirmed by sequencing.

2.3. Yeast cell transformation

For transformation experiments, the expression vector described above was linearized by cutting within the 5' AOX1 promoter fragment with SacI restriction endonuclease (Fermentas). The DNA was introduced into *P. pastoris* competent cells by electroporation as described by Lin-Cereghino et al. [42] with the aid of a MicroPulser™ Electroporator (Bio-Rad). Transformed cells were transferred to plates containing 0.67% yeast nitrogen base without amino acids, 2% glucose, 0.4 mg/l biotin and 2% agar and incubated at 30 °C for 2–3 days until colonies appeared. Transformants were checked by PCR for the presence of the GFP-*PpERG6* open reading frame. Successful expression of the GFP-PpErg6p fusion product was confirmed by fluorescence microscopy and Western blot analysis (see below).

2.4. Fluorescent microscopy

P. pastoris cells were grown on YPD medium to the early stationary phase (26 h). Cells from 1 ml culture were harvested by centrifugation, washed once with deionized water, stained for 10 min with 10 μ g/ml Nile Red (Sigma) and analyzed using a Zeiss Axiovert 35 microscope with a 100-fold oil immersion objective. Detection ranges of 450–490 nm for Nile Red and 510–520 nm for GFP were used. Images were taken with a CCD camera.

2.5. Subcellular fractionation

Lipid droplets (LD) from *P. pastoris* were obtained at high purity from cells grown to the early stationary phase as described previously [16,43] with minor modifications. Briefly, cells were grown aerobically on YPD to the early stationary phase (26 h), harvested by centrifugation and washed with deionized water. Cells were converted to spheroplasts using Zymolyase 20T (Seikagaku Corporation, Japan). Spheroplasts (~90 g) were suspended in buffer A (10 mM MES/Tris, pH 6.9, 12% Ficoll 400, 0.2 mM EDTA, 1 mM PMSF) and disintegrated using a Dounce Homogenizer (30 strokes) on ice. After centrifugation at 6,000g for 5 min the supernatant was removed and the pellet was homogenized and centrifuged again as described above. Combined supernatants (homogenate) in buffer A were centrifuged at 12,000g for 15 min. The resulting supernatant was put into an Ultra-Clear Centrifuge Tube (Beckman), overlaid with buffer A and centrifuged at 141,000g for 45 min using a swing out rotor AH-629 (Sorvall). The resulting floating layer was collected, overlaid with buffer B (10 mM MES/Tris, pH 6.9, 8% Ficoll 400, 0.2 mM EDTA, 1 mM PMSF) and ultracentrifuged at 141,000g for 30 min. The floating layer was collected again, homogenized, transferred to the bottom of the ultracentrifuge tube filled with buffer C (0.25 M sorbitol, 10 mM MES/Tris, pH 6.9, 0.2 mM EDTA) and centrifuged at 141,000g for 90 min. The resulting top layer represents highly pure lipid droplets.

Isolation of other subcellular fractions used in this study was described before [6,7,43]. For the isolation of mitochondria, microsomes and cytosol, spheroplasts (~25 g) were suspended in 10 mM Tris-HCl, pH 7.4, containing 0.6 M mannitol, homogenized with a Dounce Homogenizer (15 strokes) and centrifuged at 4,000g for 5 min. The supernatant was collected and the pellet was homogenized and centrifuged again. Combined supernatants were centrifuged at 12,000g for 15 min. The supernatant was used for microsomal and cytosol preparations (see below). The pellet was re-suspended in the same buffer and centrifuged at 4,000g for 5 min. The resulting supernatant was centrifuged at 12,000g for 10 min. The final pellet represents the mitochondria fraction. Combined supernatants from the previous steps were centrifuged at 20,000g, 30,000g and 40,000g for 30 min each. Pellet fractions of 30,000g and 40,000g centrifugation steps correspond to 30,000 g and 40,000 g microsomes. The final supernatant is cytosol including 100,000 g microsomes. The quality of subcellular fractions was routinely tested by Western blot analysis (see below).

2.6. Protein analysis

Proteins were quantified by the method of Lowry et al. [44] using bovine serum albumin as a standard. Proteins were precipitated with trichloroacetic acid and solubilized in 0.1% SDS, 0.1 M NaOH prior to quantification with the exception of LP proteins which were precipitated and de-lipidated according to the method of Wessel and Flügge [45].

SDS-PAGE was performed by the method of Laemmli [46] using 12.5% SDS gels. Samples were denaturated at 37 °C to avoid aggregation of membrane proteins. Proteins were visualized by staining with Coomassie Blue. Western blot analysis was performed according to Haid and Suissa [47]. Primary rabbit antibodies used in this study were directed against Por1p, Pma1p, Erg6p and GAPDH from S. cerevisiae, and against the 75-kDa microsomal marker protein (75-ER marker) from P. pastoris. The 75 kDa microsomal protein appears as a typical band of microsomal fractions on SDS-polyacrylamide gels. The function of this protein is unknown. For immunization the respective band was excised from a preparative SDS-polyacrylamide gel, and the protein was electro-eluted and injected into rabbits using standard procedures. Mouse antiserum against GFP was purchased from Roche. Peroxidaseconjugated secondary antibody and enhanced chemiluminescent signal detection reagents (SuperSignal[™], Pierce) were used to visualize immunoreactive bands.

2.7. Mass spectrometry of proteins

Protein (20 μ g) from the LD fraction was precipitated following the protocol of Wessel and Fluegge [45]. Dried samples were dissolved in digestion buffer (25 mM triethylammonium bicarbonate). After addition of 0.375 μ g porcine trypsin to each sample, the digestion was performed for 18 h at 37 °C with gentle agitation. The reaction was stopped by adding 1 μ l TFA (trifluoroacetic acid) to each sample. Prior to sample analysis volumes were decreased to 10 μ l by vacuum centrifugation.

Peptide separation was performed on a Proxeon Biosystems EASY-nLC[™] system coupled with a SunCollect MALDI Spotting device (SunChrom, Germany). The MALDI Spotting was done by mixing the LC-eluent with matrix solution containing 3 mg/ml alpha-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) dissolved in 70% MeCN and 0.1% TFA. The final mixture was spotted every 20 s on a blank LC-MALDI insert metal target. All MS and MS/MS were acquired on a 4800 TOF/TOF[™] Analyzer (ABSciex, Darmstadt, Germany). For protein and peptide identification an in-house Mascot database search engine v2.2.03 (Matrix Science Ltd.) and the Swissprot Protein Database were used.

2.8. Blast analysis of lipid droplet protein sequences

Amino acid sequences of *P. pastoris* LD proteins were obtained from Universal Protein Resource Knowledgebase—UniProtKB (http://www. uniprot.org/) and used for blast analysis. For the identification of orthologs from *S. cerevisiae*, the online blast tool of the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/cgi-bin/blastsgd.pl) was applied. Candidate orthologs with functions similar to those annotated for previously detected LD proteins and homologies/ similarities in amino acid sequences were selected.

2.9. Lipid extraction and analysis

Lipids from yeast cells were extracted as described by Folch et al. [48]. For quantification of non-polar lipids, extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4, Muttenz, Switzerland), and chromatograms were developed in an ascending manner by a two-step developing system. First, light petroleum/diethyl ether/acetic acid (25/25/1; per vol.) was used as mobile phase, and chromatograms were developed to half-distance of the plate. Then, plates were dried briefly and chromatograms were further developed to the top of the plate using light petroleum/diethyl ether (49/1; v/v) as the second mobile phase. To visualize separated bands, TLC plates were dipped into a charring solution

consisting of 0.63 g MnCl₂×4 H₂O, 60 ml water, 60 ml methanol and 4 ml of concentrated sulfuric acid, briefly dried, and heated at 105 °C for 40 min. Visualized lipids together with ergosterol, triolein and cholesteryl ester as standards were quantified by densitometric scanning at 400 and 600 nm using a Shimadzu scanner CS-930.

For phospholipid analysis, lipids from homogenate and LD were loaded on silica gel 60 plates (Merck, Darmstadt, Germany), separated by two dimensional TLC (2D-TLC) using chloroform/methanol/25%NH₃ (65/35/5; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50/20/10/10/5; per vol.) as second developing solvent system. Phospholipids were stained with iodine vapor, scraped off and quantified by the method of Broekhyuse [49]. The same method was applied for quantification of total phospholipids obtained as bands from one dimensional TLC using light petroleum/diethyl ether/acetic acid (25/25/1; per volume) as developing system.

Fatty acids were analyzed by gas–liquid chromatography (GLC). Lipid extracts prepared as described above were subjected to methanolysis using 2.5% H_2SO_4 in methanol and converted to methyl esters. Fatty acid methyl esters were separated using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-INNOWax capillary column (15 m×0.25 mm inner diameter×0.50 µm film thickness) and helium as carrier gas. Fatty acids were identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN).

Individual sterols from total cell extracts (homogenates) or LD were identified and quantified by GLC/MS after alkaline hydrolysis of lipid extracts [50]. GLC/MS was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a mass selective detector (HP 5972), using an HP5-MS capillary column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ µm}$ film thickness). Aliquots of 1 µl were injected in the splitless mode at 270 °C injection temperature with helium as a carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100 °C, 10 °C/min to 250 °C, and 3 °C/min to 310 °C. Mass spectra were acquired in scan mode (scan range 200–550 atomic mass units) with 3.27 scans/s. Sterols were identified based on their mass fragmentation pattern.

2.10. Lipidome analysis by LC-MS

Lipids were extracted as described above and dissolved in chloroform/ methanol (1/1; v/v) spiked with a set of 28 internal standards. Internal standardization and data acquisition by HPLC coupled to a FT-ICR-MS hybrid mass spectrometer (LTQ-FT, Thermo Scientific) was described previously in much detail by Fauland et al. [51]. Data processing takes into account exact mass and retention time and was performed by Lipid Data Analyzer according to Hartler et al. [52].

3. Results

3.1. Isolation of lipid droplets from P. pastoris and quality control

Lipid droplets (LD) are lipid storage compartments present in all types of eukaryotic cells including various yeast species. Here we describe isolation and characterization of LD from the industrial yeast *P. pastoris* to broaden our fundamental knowledge of the cell biology and the lipid metabolism of this important microorganism. For the isolation of LD from *P. pastoris* cells we adapted and optimized a protocol which had been established previously for *S. cerevisiae* [16]. The procedure is described in detail in Experimental procedures section.

For the quality control of isolated LD from *P. pastoris* cellular fractions were separated by SDS–polyacrylamide electrophoresis (Fig. 1A) and tested by Western blot analysis (Fig. 1B). As marker protein for *P. pastoris* LD we used Erg6p and Erg6–GFP. Localization of the Erg6–GFP-hybrid protein to LD was confirmed by fluorescence microscopy and co-staining with Nile Red (Fig. 1C). Western blot analysis (see Fig. 1B) revealed high enrichment of Erg6p and GFP-Erg6p in

isolated LD fractions. Not surprisingly, the overexpressed GFP-Erg6 hybrid was also found in microsomal fractions at lower concentration as had been shown previously for Erg6p in *S. cerevisiae* [24]. Cross-contamination of *P. pastoris* LD with other subcellular fractions was only marginal. Hence, the isolation protocol was efficient and could be used as the basis for biochemical characterization of *P. pastoris* LD.

3.2. Non-polar storage lipids of lipid droplets from P. pastoris

Non-polar lipids are the major components of LD. In yeast, nonpolar lipids accumulate at substantial amounts in the stationary growth phase. For this reason, *P. pastoris* cells grown on glucose were harvested at the early stationary phase (26 h) and LD were analyzed. TG (~55–65 mg/mg LD protein) comprised more than 90% of all non-polar lipids from LD. The amount of SE (~3–5 mg/mg LD protein) was low resulting in an approximate TG to SE ratio of ~15. Only traces of free sterols were detected in LD. This finding is in sharp contrast to LD from *S. cerevisiae* where SE and TG are present at equal amounts [16]. However, the high TG to SE ratio in *P. pastoris* is reminiscent to LD from the yeast *Yarrowia lipolytica* [53] but also to mammalian adipocytes [54].

Whereas in *S. cerevisiae* the excess of sterols formed is converted to substantial amounts of SE stored in LD [36,37] the obviously small amount of sterols formed in *P. pastoris* appears to be the reason for moderate occurrence of SE. However, accumulation of ergosterol precursors in LD from *P. pastoris* is similar to *S. cerevisiae*. As can be seen from Fig. 2, the sterol composition of *P. pastoris* LD mainly derived from the esterified form as SE is completely different from total cell extracts. In homogenates, the final product of the sterol biosynthetic pathway, ergosterol, is strongly enriched and comprises up to 80% of total sterols. Only small amounts of ergosterol precursors were found in total cell extracts. In contrast, LD contain ergosterol only at 30% of total sterol, but sterol intermediates are present at large quantities. In LD, the concentration of zymosterol is comparable to ergosterol (~26%), and also episterol, 4-methylzymosterol, fecosterol, episterol, lanosterol and 4,14-dimethylcholesta-8,24-dienol are strongly enriched over the homogenate.

3.3. Phospholipid analysis

A phospholipid monolayer forms the surface membrane of LD and shields the highly hydrophobic particle from the aqueous environment. This surface membrane is assumed to be very important for the maintenance and the structure of LD [55]. Fig. 3 shows a comparison of phospholipid compositions from *P. pastoris* cell homogenate and LD. First, the ratio of phospholipids to proteins was found to be very high in LD which is, however, mainly result of the low abundance of proteins in LD (Fig. 3A). The ratio of TG to total phospholipids was ~60 indicating the low abundance of the latter lipid class in LD and at the same time confirming the high purity of isolated LD fractions. The pattern of major phospholipids in LD roughly reflected total cell extracts, although certain differences are noteworthy (Fig. 3B). Whereas phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are major phospholipids of both LD and homogenate, the ratio of phosphatidylinositol (PI) to phosphatidylserine (PS) differs strongly. This PI to PS ratio is ~1 in the homogenate and ~7.5 in LD. Other phospholipid species such as cardiolipin (CL), phosphatidic acid (PA), lysophosphatidic acid (LPA) and dimethyl-phosphatidylethanolamine (DMPE) were only present at trace amounts in LD.

Besides conventional phospholipid analysis we extended our studies to mass spectrometric analysis of PC, PE, PI and PS (Fig. 4). This analysis revealed that there were only minor differences in the



Fig. 1. Quality control of isolated lipid droplets. A–Protein pattern of lipid droplet fraction and control fractions (10 µg protein). Homogenate (H), lipid droplets (LD), 30,000 g microsomes (30 m), 40,000 g microsomes (40 m), cytosol (C) and mitochondria (M) fractions were loaded onto an SDS–polyacrylamide gel. B–Western blot analysis. Antisera against *Pichia pastoris* 75-ER marker protein (microsomal marker); Pma1p, plasma membrane H⁺-ATPase (plasma membrane marker); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (cytosolic marker); GFP-Erg6p, green fluorescent protein fused to Erg6p (LD marker); Erg6p, Δ(24)-sterol C-methyltransferase (LD marker); and Por1p, mitochondrial porin (mitochondrial marker) were applied. C–Fluorescent imaging. Cells were grown on glucose at 30 °C for 26 h, stained with Nile Red and subjected to fluorescent microscopy. The Erg6p-GFP-signal coincides with Nile Red stained LD.



Fig. 2. Sterol composition of lipid droplets. Cells were grown on glucose at 30 °C to the stationary phase (26 h). Lipid extracts from LD (white bars) and homogenates (grey bars) were subjected to MS analysis of sterols. Amounts of individual sterols are shown as percentage of total sterols. Zym, Zymosterol; Erg, ergosterol; m-Zym, 4-methylzymosterol; Fec, fecosterol; Epi, episterol; Lan, lanosterol; DMCD, 4,14-dimethylcholesta-8,24-dienol. Data are mean values of three independent experiments. Error bars indicate standard deviation.

phospholipid species patterns of homogenate and LD. The vast majority of all phospholipid species in both samples comprised C36 and C34 species. The fact that the entire phospholipid species profile of LD follows the homogenate supports the notion postulated in a study with *S. cerevisiae* [18] that there is no species selective transfer of phospholipids to LD. This result also suggests that proteins of the LD surface membrane do not require a specific phospholipid species milieu. However, the difference in the PI/PS ratio as mentioned above has to be kept in mind.

When examining individual phospholipids of *P. pastoris* homogenate and LD fractions we observed peculiar properties of the different phospholipid classes. PC is the phospholipid with the largest variety of species in both homogenates and LD. Noteworthy, PC occurs in the form of C36:2 and several polyunsaturated species such as C36:3, C36:4 and C36:5. The species pattern of PE is markedly different from PC. The most prominent PE species are C36:2, C34:1 and C34:2. Interestingly, in both homogenate and LD PI is presented mostly as C34:1 and C34:2 species. The simplest species pattern was observed with PS being restricted to C34:1 and C34:2. Similar results were obtained with *S. cerevisiae* LD where C34:1 was the major PI species and PS was mostly present in its C34:1 and C34:2 form [18].

3.4. Fatty acids analysis of lipid droplets

The vast majority of fatty acids in LD are stored in the form of TG and SE and only a minor part is present in the surface phospholipid monolayer. To address the specificity of the lipid storage machinery in P. pastoris we analyzed the fatty acid composition of LD. As can be seen from Fig. 5, oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and palmitic (C16.0) acids are dominant in both LD and homogenate. Other fatty acids are present only at minor amounts in both samples. This finding indicates that in *P. pastoris* the distribution of fatty acid species is not specific for LD and reflects the total cell fatty acid pattern. We can exclude from these data that *P. pastoris* enzymes synthesizing non-polar lipids have strong fatty acid substrate specificity. This result is in contrast to the two TG synthases from S. cerevisiae, Dga1p and Lro1p, which have a clear preference for unsaturated fatty acids, especially C16:1 and C18:1 [18,56]. With that respect, P. pastoris LD are different from other yeasts such as S. cerevisiae [18] and Y. lipolytica [53], but similar to plant oil bodies which also contain marked amounts of polyunsaturated fatty acids [57].

3.5. P. pastoris lipid droplet proteome

To complete the characterization of *P. pastoris* LD we performed a proteome analysis of this compartment. For this investigation we utilized direct MS as outlined in detail in Experimental procedures section. Proteins were identified through annotation in the Universal Protein Resource Knowledgebase (http://www.uniprot.org/) and assigned to putative functions according to blast and motif searches. Additionally, we identified *S. cerevisiae* orthologs with their cellular localization and biological functions. Data are summarized in Table 1.

The function of most *P. pastoris* LD proteins identified here is hypothetical and derived from bioinformatic analysis (http://www.uniprot.org/20121203). However, detected proteins can be divided in two groups depending on association with lipid metabolism or being involved in other cellular processes. The first group includes the most abundant proteins of the *P. pastoris* LD proteome such as enzymes involved in ergosterol, phospholipid and sphingolipid biosynthesis as well as fatty acid metabolism, fatty acid transport and activation, and lipolysis. These findings are in line with proteomic studies of *S. cerevisiae* LD performed recently in our laboratory [18]. The second group of *P. pastoris* LD proteins identified here is not associated with lipid metabolism, but with ribosomal translation, energy



Fig. 3. Phospholipid pattern of lipid droplets. Cells were grown on glucose at 30 °C to stationary phase (26 h). Lipid extracts were analyzed for phospholipids as described in the experimental section. A—Total phospholipids in LD and homogenate. B—Relative distribution of individual phospholipids in LD (white bars) and homogenates (grey bars). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), phosphatidic acid (PA), lysophospholipids (LP), dimethylphosphatidylethanolamine (DMPE), are shown as percentage of total phospholipids. Data are mean values of at least three independent experiments. Error bars indicate standard deviation.



Fig. 4. Phospholipid species composition. Lipid extracts from homogenate (grey bars) and LD (white bars) were analyzed by LC–MS for phospholipid species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

metabolism and mitochondria function. Although our LD preparations from *P. pastoris* were shown to be of high quality, contamination with other subcellular fractions cannot be completely excluded. However, recent studies showed structural and functional interactions between LD and other organelles [58–62] which are of physiological relevance. The possible involvement of proteins found in this study in such processes will be subject to individual investigations.

4. Discussion

During the investigation presented here we established a reliable technique for the isolation of highly pure LD from the industrial



Fig. 5. Fatty acid pattern of lipid droplets. Lipid extracts from LD (white bars) and homogenate (grey bars) were analyzed by GC-FID for fatty acid composition. The amounts of individual fatty acids in homogenate and LD are shown as % of total fatty acids. Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

yeast *P. pastoris* which enabled us to analyze lipidome and proteome of this organelle. Our results confirm in general our current knowledge of yeast LD biochemistry, although several peculiarities in lipid and protein composition of LD from *P. pastoris* were observed. A specific and remarkable feature of *P. pastoris* LD is the high prevalence for TG over SE in its hydrophobic core. This result is in strong contrast to *S. cerevisiae* LD which contain equivalent amounts of TG and SE [16]. The other difference between *P. pastoris* and *S. cerevisiae* LD is the unselective occurrence of poly/unsaturated fatty acids in the former microorganism. This finding is in line with our studies of other *P. pastoris* organelles, e.g. with peroxisomes and mitochondria [5,6], which did not show any specific fatty acid targeting either. Thus, the high degree of fatty acid unsaturation appears to apply to most *P. pastoris* organelles.

Proteome analysis of P. pastoris LD revealed a distinct set of LD associated proteins. Based on homologies to S. cerevisiae a number of these proteins were identified. However, functions of some of these newly identified proteins remained unassigned. Interestingly, the total number of P. pastoris LD proteins found in this study was rather low compared to investigations with S. cerevisiae [18], mammalian cells [59,63,64] and plant cells [65-67]. We can speculate that P. pastoris LD require only a minimal set of proteins to maintain structure and function of this organelle, and that functions of LD from this yeast are not diverse from other organisms [68-70]. Our results also demonstrate that LD from P. pastoris contribute to lipid metabolism (see Table 1) as has been shown before for LD from other cell types. Individual features or functions of LD from different species may be explained by differences in the proteome. From this view point "unusual" or unidentified proteins in the LP proteome from P. pastoris may become important. The functional relevance of such proteins will have to be tested rather on an individual than on a global basis. Proteome analysis as presented here may set the stage for such studies.

Table 1

Proteome of *P. pastoris* lipid droplets. LD were isolated from cells grown on glucose to stationary phase (26 h, 30 °C). Proteins were subjected to MS analysis (see Experimental procedures). Blast analysis of amino acid sequences revealed *S. cerevisiae* orthologs and the degree of homology. Localization and biological processes inferred from homology are shown. Databases used were Universal Protein Resource Knowledgebase–UniProtKB (http://www.uniprot.org/20121203) and Saccharomyces Genome Database–SGD (http://www.yeastgenome.org/20121203). C, cytosol; M, mitochondria; PM, plasma membrane; ER, endoplasmic reticulum; LD, lipid droplets; E, endosomes; G, Golgi, N, nucleus; R, ribosome.

C484.03Defail 24information C-nethydransferazeFg/0g (73)FKLDMEngoserol housynthetic processC48118Jong Chain faty 2-01 CAS synthetas with acidsFg/0g (73)FKLDMErgoserol housynthetic processC48208Syntheta Poix/Sac. Calibyes the poix/Sa	UniProtKB ID	Submitted name (UniProtKB)	S. cerevisiae ortholog (homology %)	S. cerevisiae ortholog localization (SGD)	Biological process inferred from homology (SGD)
CARLERSLong chain futy apt-CaA synthetase with a part [530]LDAWPMLong-chain struy apt-capport, long-chain faty-apt-CAA metabolic processCARDARSpalene pot.CAS structuresFig1 [533]ERLDSterol metabolic processCARDARSpalene to 23-action structureTable (533)CRRM3-Keto-sphinganine metabolic processCARDAR3-Keto-sphinganine metabolic processTable (243)LDMMedium-chain fary add biosynthetic processCARDAR3-Keto-sphinganine metabolic processTable (243)LDMMedium-chain fary add biosynthetic processCARDARand EntripTable (243)LDMMedium-chain fary add biosynthetic processCARDARcatalyces the optication of spalene 23-spondeERLD/PMEgestref biotoses, sterol metabolic processCARDARcatalyces the optication of spalene 23-spondeERLD/PMEgestref biotoses, cell rode holysynthetic (ratabolic processCARDARand EntripPath (233)LDARCARDARcatalyces the optication of spalene 24-spondeEggtref biotoses, cell rode holysynthetic (ratabolic processCARDARand EntripPath (233)RCrossende (1640)CARDARConserver (1080)Path (233)RCrossende (1640)CARDARNot formUnknownUnknownCARDARNot formUnknownApoptolic processCARDARNot formUnknownApoptolic processCARDARNot formUnknownApoptolic processCARDARNot formUnknownApoptolic process	C4R4C9	Delta(24)-sterol C-methyltransferase	Erg6p (67%)	ER/LD/M	Ergosterol biosynthetic process
reference for 12:0-C1:6:0 farly acidsFuel (537)EVLDExtrol metabolic processC4R121Squalene (poxidae, calityes the qoxidae)Tc10 (387)C/ER/M3-Kero-sphinganine metabolic process, sphingalipidC4R24A/Fenorphingkine fueltase, catabyes the goxidaeTc10 (387)LD/MKeldue-chain farty acid biosynthetic/catabolic processC4R032Asynthesis (1997)LD/MCellular injerate abolic process, sterol metabolic processEgositerol biosynthetic/catabolic processC4R033Steryl exter hydrolaseExt (1977)LD/MKeldue-chain farty acid biosynthetic/catabolic processC4R034Asynthesis (1997)Patabolic process (1997)Egositerol biosynthetic/catabolic processC4R034Asynthesis (1997)RelLD/PMEgositerol biosynthetic/catabolic processC4R044Patabolic process (1997)RelLD/PMTranslation (RNA binding)C4R045Conserved rhosonal protein VIA326WYI326wp (283)UnknownUnknownC4R124Michondrial Jorin (1991)No foundUnknownUnknownCroplasmic translation, rhosonal large subunitC4R124Nachondrial Jorin (1991)No foundUnknownUnknownUnknownC4R124LAppinerized methabae proteinNo foundUnknownUnknownC4R125Asynthesis (1997)MConserved rhosonal large subunitC4R124LAppinerized proteinNo foundUnknownUnknownC4R125Asynthesis (1997)MConserved rhosonal large subunitC4R126No foundNo foundUnknown <t< td=""><td>C4R1R9</td><td>Long chain fatty acyl-CoA synthetase with a</td><td>Faa1p (59%)</td><td>LD/M/PM</td><td>Long-chain fatty acid transport, long-chain</td></t<>	C4R1R9	Long chain fatty acyl-CoA synthetase with a	Faa1p (59%)	LD/M/PM	Long-chain fatty acid transport, long-chain
C4R3L8Squalence to 2-voldsqualerErg1p (53%)ER/L0Stroll metabolismC4QX663-ketrosphinganine reductase, ctalyzes the ordsgrave to dep in plytophingonie synthesisTarl(p (38%)C/ER/M3-ketro-sphinganine reductase, ctalyzes the ordsgrave to dep in plytophingonie synthesisC4QX64Acyl-oenzymek-rethanOl a-glytansferase to ansetrol synthase, an essential enzyme that tarlyzes the cylitation of squaler 2-sprave tarlyzes the cylitation sprave to the small (405) ribosomal targes tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of the small (405) ribosomal targe tarlyzes the cylitation of tarlyzes the cylitation of tarlyzes the cylitation of tarlyzes the cylitation (405) ribosomal targes tarlyzes the cyl		preference for C12:0-C16:0 fatty acids			fatty-acyl-CoA metabolic process
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C4Q24 Acy-consymmeX-fethanol C-syltransferate Eht [p (37%) D/M Medium-chain stay acd biosynthetic/catabolic process C4R403 Service jester hydrohase Tg1 (42) LD Cellular lipid metabolic process C4R403 Service jester hydrohase Tg1 (42) EU/LP/M Egosterol biosynthetic/catabolic process C4R403 Autative acyltransferase with similarity to EeDi EeDi (35%). EU/LP/M Edium-chain atty acd biosynthetic/catabolic process C4QVA2 Protein component of the small (405) ribosoma Rp30 (82%) R Translation (RAM binding) F2QD30 Uncharacterized membrane protein YLR326W YIF326W (28%) Unknown Unknown C4QV54 Enserved ribosomal protein YLR326W YIF326W (28%) M Apoptotic process C4R12 Mitchendrala protein YLR326W YIF326W (28%) M Apoptotic process C4QV54 Laudev uncharacterized protein reserver Not found Unknown Cytoplasmic translation, ribosomal large subunit C4QV54 Laudev uncharacterized protein reserver St1 (50%) LD Olytoplasmic translation, ribosomal large subunit C4QV54 Laudev uncharacterized protein reserver St1 (50%) LD Olytoplasmic translation, ribosomal large subunit C4QV54 Laudev uncharacterized protein subunit Krg7 (60%) M		second step in phytosphingosine synthesis			biosynthetic process
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controlburnan PD, and E. coli L10eassembly, translational elongationCRR122Minochondria point (Voltage-dependent anion chamel), outer membrane proteinPor1p (473)MAppolotic process, cell redox homeostasis, transport, ion transport, mitochondrion organizationC4QVF8Patative uncharacterized protein 3 keto streol reductaseNot foundUnknownUnknownC4QVF81-Acyl-sm-glycerol-3-phophate acyltransferase streol reductaseSIC (p (53)ER/MErgostreol biosynthetic processC4R205ATP synthass couplied proton transport reductaseFig27p (633)R/CTranslational elongation, RNA export from nucleusC4QVE0Elongation factor 1-alphaTel (p (83), FATY softhase scupiled proton transportRCTranslational elongation, RNA export from nucleusC4QVE0Fatty acid transport and very long-chain fatty acid-CaS synthesizeFattp (493)ER/LD/P/MLong-chain fatty acid transport, very long chain fatty acid transport acid elongation acid biosynthetic processC4QVE0Patative acharadivex yacetone phosphate reductaseHifd Ip (433)ER/LD/C/MCellular aldehyde metabolic processC4QV007Steryl ester hydrolase, one of three gene products (Yeh Ip, Yeh2p, Tel Ip)Tgl (p (33), RCCellular aldehyde metabolic processC4QV07Streyl ester hydrolase, one of three gene products (Yeh Ip, Yeh2p, Tel Ip)Opi Ip (272)ER/NLong-chain fatty acid transport, expl one chain fatty acid transport and acid biosynthetic processC4QV04Streyl ester hydrolase, one of three gene products (Yeh Ip, Yeh2p, Tel Ip)Opi Ip (272	C4QV50	Conserved ribosomal protein PO similar to rat PO,	Rpp0p (71%)	R	Cytoplasmic translation, ribosomal large subunit
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channel), outer membrane proteinNot foundUnknowntransport, mitochondrion organizationC4QVF81-AqJ-sn-glycerol-3-phosphate acyltransferaseSIC Jp (50%)LDGlycerophospholipid biosynthetic processC4R1963-Keto sterol reductaseSIC Jp (50%)ER/MErgosterol biosynthetic processC4R205ATP synthase subunit betaAtp2 (87%)MATP synthase iscoupled proton transportC4Q206Elongation factor 1-alphaTeTp (88%)MATP synthase iscoupled proton transportC4Q206Fatty acid transporter and very long-chain fatty acyl-CoA synthetaseFattp (49%)ER/LD/MCellular aldehyde metabolic processC4Q207Fatty acid transporter and very long-chain fatty acyl-CoA synthetaseHfI p (43%)E/LD/MCellular aldehyde metabolic processC4Q207Putative fatty aldehyde dehydrogenaseHfI p (43%)E/LD/MCellular lipid metabolic processC4Q207Steryl ester hydrolase, one of three gene phosphate reductaseTgI p (39%)LDCellular lipid metabolic processC4Q007Steryl ester hydrolase, one of three gene products (vell-hy (vel2), TgI p)Protein involved in ER-associated protein torocesNot posphate foundaseC4Q008Transcriptional repressor OP11Opi p (27%)ER/NUnfolded protein response, regulation of transcription, phospholiphid biosynthetic processC4QV54Protein involved in ER-associated protein torier metaboreEnv9 (48%)RCytoplasmic translationC4QV50Afriboradin lipid particlesEnv9 (48%)RCytoplasmic transl	C4R1Z2	Mitochondrial porin (Voltage-dependent anion	Por1p (47%)	М	Apoptotic process, cell redox homeostasis, transport, ion
C4QVF8 C4QVF8Putative uncharacterized proteinNot foundUnknownUnknownC4QVF8 C4QVF41-Acyls-mg/tycerol-3-phosphalipid biosynthetic processSIC10 (SV)LDClycerophospholipid biosynthetic processC4R196 C4R2N53-Keto sterol reductaseErg27p (63%)R/CTranslational elongation, tRNA export from nucleusC4Q2B0 C4R2N5Elongation factor 1-alphaAtp2 (R3%)MATP synthases coubult departed transportC4Q2D6 C4R2N5ATP synthase subunit alphaTef1p (89%)R/CTranslational elongation, tRNA export from nucleusC4R2N5ATP synthase subunit alphaTef1p (89%)MATP synthasis coupled proton transport.C4R2N5ATP synthase subunit alphaFat1p (43%)ER/LD/PMLong-chain fat1y add transport. very long chain faty add metabolic processC4QXD6Putative fat1y aldehyde dehydrogenaseHfd1p (43%)ER/LD/CMPhosphatidic acid biosynthetic processC4QXD7Steryl ester hydrolase, one of three gene products (verh hty, Veh2p, Tg1p)Tg1p (39%)LDCellular lipid metabolic process, sterol metabolic processC4QVN0Nop-ADP/ATP carrier of the mitochondrial inner membranePet9p (85%)MRespirationF2QVQ8Transcriptional repressor OP1Op1ip (27%)ER/NUnfolded protein response, regulation of transcription, phosphatific brocess, proteasomal protein indeprications, proteosin deprications, proteosin deprications, protein involved in ER-associated proteinNu Sp2 (25%)RC4QVN4405 ribosomal protein subunitRp29ap (88%)RSt		channel), outer membrane protein			transport, mitochondrion organization
C4QVF41-Acyt-sn-glycerol-3-phosphate acyltransferaseSich (p 50%)LDGlycerophospholipid biosynthetic processC4R1963-keto sterol reductaseErg276 (53%)ER/MErgostron biosynthetic processC4R200Elongation factor 1-alphaTefp (89%)R/CTranslational elongation, tRNA export from nucleusC4Q200Elongation factor 1-alphaTefp (89%)R/CTranslational elongation, tRNA export from nucleusC4Q206Fatty acid transporter and very long-chain fatty acid metabolic processHalp (88%)MATP synthesis coupled proton transportC4Q207Putative fatty aldehyde dehydrogenaseHfl1p (43%)E/LD/MCellular aldehyde metabolic processC4Q207Steryl ester hydrolase, one of three geneTgl1p (39%)LDCellular lipid metabolic processC4Q208Transcriptional represor OP1Op1p (27%)ER/LD/C/MPhosphatidic acid biosynthetic processC4QW07Steryl ester hydrolase, one of three geneTgl1p (39%)LDCellular lipid metabolic process, sterol metabolic processC4QW08Transcriptional represor OP1Op1p (27%)ER/MEesprationC4QW21Protein involved in ER-associated proteinUb2p (25%)ER/MER-associated protein crabolic process, proteasonal degradationC4QW2440S ribosomal protein subunitRps2ap (84%)RCytoplasmic translationC4QW24Protein involved in ER-associated proteinUb2p (25%)ER/MER-associated protein represor, proteasonal protein degradations, protein secretionC4QW24Protein invol	C4QVF8	Putative uncharacterized protein	Not found	Unknown	Unknown
C4R1963-Keto sterol reductaseErg27p (63%)ER/MErgosterol biosynthetic processC4R2N5ATP synthase subunit betaAtp2 (67%)MATP synthesis coupled proton transportC4Q2D6Elongation factor 1-alphaTeTp (98%)R/CTranslational elongation, tRNA export from nucleusC4R4Y8ATP synthase subunit alphaAtp1 (88%)MATP synthesis coupled proton transportC4Q2N6Fatty acid transporter and very long-chain fattyFat1p (49%)ER/LD/PMLong-chain fatty acid transport, very long chain fatty acid metabolic processC4Q2X16Putative fatty aldehyde dehydrogenaseHfd1p (43%)E/LD/C/MPhosphatidic acid biosynthetic processC4Q2X26NADPH-dependent 1-acyl dihydroxyacetoneAyr1p (52%)ER/LD/C/MPhosphatidic acid biosynthetic processC4Q2W07Steryl ester hydrolase, one of three gene products (Yeh1p, Yeh2p, Tg1p)Tg1p (39%)LDCellular lipid metabolic process, sterol metabolic processC4Q2W04Major ADP/ATP carrier of the mitochondrial inner membranePet9p (85%)MRespirationF2QNQ8Transcriptional repressor OP11Opi1p (27%)ER/MLnfolded protein response, regulation of transcription, phospholipid biosynthetic process, proteasomal gerzationC4Q2W1440S ribosomal protein subunitRps2apa (88%)RCytoplasmic translationC4Q2W2Protein inviho with similarity to oxidoreductases, found in lipid particlesEnv9 (48%)RStructural constituent of ribosomeC4Q2W1440S ribosomal protein S0 nearly identical to kps0bp <td>C4QVE4</td> <td>1-Acyl-sn-glycerol-3-phosphate acyltransferase</td> <td>Slc1p (50%)</td> <td>LD</td> <td>Glycerophospholipid biosynthetic process</td>	C4QVE4	1-Acyl-sn-glycerol-3-phosphate acyltransferase	Slc1p (50%)	LD	Glycerophospholipid biosynthetic process
C4R25 APP synthase subunit beta Atp2p (87%) M APP synthesis coupled proton transport (4Q250 Elongation factor 1-alpha Tefp (89%) R/C Translational elongation (RNA export from nucleus ATP synthesis coupled proton transport (4Q250 Elongation factor 1-alpha Atp1p (89%) R/C Translational elongation (RNA export from nucleus actional elongation factor 1-alpha (47%) EAUD/PM Long-chain fatty acid transport, very long chain fatty acid transport, erg long chain fatty acid transport, erg long chain fatty acid transport erg long chain fatty acid transport, erg long chain	C4R196	3-Keto sterol reductase	Erg27p (63%)	ER/M	Ergosterol biosynthetic process
C4Q280Elongation factor 1-alphaTelTp (89%)R/CTranslational elongation, itNA export from nucleusC4R4Y8ATP synthase subunit alphaAtp1 (88%)MATP synthesis coupled proton transportC4QXD6Fatty acid transporter and very long-chain fatty acyl-CoA synthetaseFat1p (49%)EK/LD/PMLong-chain fatty acid transport, very long chain fatty acid metabolic processC4QXC1Putative fatty adehyde dehydrogenaseHfd1p (43%)E/LD/MCellular aldehyde metabolic processC4QW07Steryl ester hydrolase, one of three gene products (Yeh1p, Yeh2p, Tg11p)Tg11p (39%)LDCellular lipid metabolic process, sterol metabolic processC4QW07Steryl ester hydrolase, one of three gene products (Yeh1p, Yeh2p, Tg11p)Pet9p (85%)MRespirationC4R760Major ADP/ATP carrier of the mitochondrial inner membranePet9p (85%)RCytopalsmic translationC4QW44405 ribosomal protein subunitRps2gap (88%)RCytopalsmic translationC4QW21Protein involved in ER-associated protein to mith similarity to oxidoreductases, to Rps0BpEn/9 (48%)LDVacual protein process, proteasomal protein degradation, grotein science (39%)C4R7K7Protein involved in ER-associated protein to Rps0BpTom5p (39%)RStructural constituent of ribosomeC4QYK4406 ribosomal protein S0 nearly identical to Rps0BpRps0ap (84%)RStructural constituent of ribosomeC4QYK4406 ribosomal protein to Rps0BpTom5p (39%)MProtein intageting to mitochondrionC	C4R2N5	ATP synthase subunit beta	Atp2p (87%)	M	ATP synthesis coupled proton transport
C4R4Y8AIP synthase subunit appaApp (98%)MAIP synthesis coupled proton transportC4QXD6Fatty acid transporter and very long-chain fatty acid metabolic processFatty (49%)EK/LD/PMLong-chain fatty acid transportC4QXD6Putative fatty aldehyde dehydrogenaseHfd1p (43%)E/LD/MCellular aldehyde metabolic processC4QXD7Putative fatty aldehyde dehydrogenaseHfd1p (43%)E/LD/MPhosphatific acid biosynthetic processC4QX07Steryl ester hydrolase, one of three gene products (Yeh1p, Yeh2p, Tgl1p)Tgl1p (39%)LDCellular lipid metabolic process, sterol metabolic processC4R760Major ADP/ATP carrier of the mitochondrial inner membranePet9p (85%)MRespirationF2QNQ8Transcriptional repressor OP11Opi1p (27%)ER/NUnfolded protein response, regulation of transcription, phospholipid biosynthetic processC4QV1440S ribosomal protein subunitRps29ap (88%)RCytoplasmic translationC4QV12Protein involved in ER-associated proteinUbx2p (25%)ER/MER-associated protein catabolic process, proteasomal degradationC4R7R7Protein with similarity to oxidoreductases, to Rys0BpEnvyla (48%)RStructural constituent of ribosomeC4QV50Photephotephotephotephotephotephotephotep	C4QZB0	Elongation factor 1-alpha	Tef1p (89%)	R/C	Translational elongation, tRNA export from nucleus
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C4R226NADPH-dependent 1-acyl dihydroxyacetone phosphate reductaseAyr1p (52%)ER/LD/C/MPhosphatidic acid biosynthetic processC4QW07Steryl ester hydrolase, one of three gene products (Yeh1p, Yeh2p, Tg11p)Tg11p (39%)LDCellular lipid metabolic process, sterol metabolic processC4R760Major ADP/ATP carrier of the mitochondrial inner membranePet9p (85%)MRespirationF2QNQ8Transcriptional repressor OPI1Op11p (27%)ER/NUnfolded protein response, regulation of transcription, phospholipid biosynthetic processC4QV1440S ribosomal protein subunitRps29ap (88%)RCytoplasmic translationC4QV14Protein involved in ER-associated protein degradationUbX2p (25%)ER/MER-associated protein catabolic process, proteasomal protein degradations, protein secretionC4R7R7Protein with similarity to oxidoreductases, found in lipid particlesEnv9p (48%)LDVacuolar protein processing, vacuole organizationC4QYK040S ribosomal protein S0 nearly identical to Rps08pRps0ap (84%)RStructural constituent of ribosomeC4QYK19Platative uncharacterized protein out of the cellTom5p (39%)MProtein targeting to mitochondrionC4QVQ4AN1-type zinc finger protein YNL155W TATISSWYin150xp (30%)C/NUnkownC4QV405Protein inport into YNL155WYin150xp (30%)C/NUnkownC4QV405GTPase, similar to Ypt51p and Ypt53p and to marmalian rab5Vps21p (54%)EEEndocytosis, protein targeting to vacuole folding </td <td>C4QXC1</td> <td>Putative fatty aldehyde dehydrogenase</td> <td>Hfd1p (43%)</td> <td>E/LD/M</td> <td>Cellular aldehyde metabolic process</td>	C4QXC1	Putative fatty aldehyde dehydrogenase	Hfd1p (43%)	E/LD/M	Cellular aldehyde metabolic process
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C4R760Major ADP/ATP carrier of the mitochondrial inner membranePet9p (85%)MRespirationF2QNQ8Transcriptional repressor OP11Opi1p (27%)ER/NUnfolded protein response, regulation of transcription, phospholipid biosynthetic processC4QYN440S ribosomal protein subunitRps29ap (88%)RCytoplasmic translationC4QW21Protein involved in ER-associated proteinUbx2p (25%)ER/MER-associated protein catabolic process, proteasomal protein degradation, protein soluentic soluentic processC4R7R7Protein with similarity to oxidoreductases, 	C4QW07	Steryl ester hydrolase, one of three gene	Tgl1p (39%)	LD	Cellular lipid metabolic process, sterol metabolic process
Finder MarkerField (GSP)ImRespirationF2QNQ8Transcriptional repressor OPI1Opi1p (27%)ER/NUnfolded protein response, regulation of transcription, phospholipid biosynthetic processC4QVN440S ribosomal protein subunitRps29ap (88%)RCytoplasmic translationC4QW21Protein involved in ER-associated proteinUbx2p (25%)ER/MER-associated protein catabolic process, proteasomal degradationC4R7R7Protein involved in Sinilarity to oxidoreductases, found in lipid particlesEnv9p (48%)LDVacuolar protein processing, vacuole organization found in lipid particlesC4QYK040S ribosomal protein S0 nearly identicalRps0ap (84%)RStructural constituent of ribosome to Rps0BpC4R7R7Putative uncharacterized proteinTom5p (39%)MProtein targeting to mitochondrionC4QVS040S ribosomal protein S0 nearly identicalRps0ap (84%)RStructural constituent of ribosome to Rps0BpC4R7L9Putative uncharacterized proteinTom5p (39%)MProtein targeting to mitochondrionC4QVS0Plasma membrane H ⁺ -ATPase, pumps protonsPma1p (86%)PMPrototin transport, regulation of pHcut of the cellF2QYU4AN1-type zinc finger protein YNL155WYnl155wp (30%)C/NUnknownC4QXL9GTPase, similar to Ypt51p and Ypt53p and toVps21p (54%)EEndocytosis, protein targeting to vacuoleC4QW26Prenyltransferase, required for cell viabilityNus1p (47%)LD/ERProtein glycosylationC4QW26Prenyltransferase, requi	C4R760	Major ADP/ATP carrier of the mitochondrial	Pet9n (85%)	М	Respiration
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C4R7R7Protein with similarity to oxidoreductases, found in lipid particlesEnv9p (48%)LDVacuolar protein recreating vacuole organization found in lipid particlesC4QYK040S ribosomal protein S0 nearly identical 	0401/21	degradation	ODX2p (23%)		protein degradations, protein secretion
Found in lipid particlesEnerg (CB)Energ (CB)Energ (CB)Energ (CB)Energ (CB)C4QYK040S ribosomal protein S0 nearly identical to Rps0BpRps0ap (84%)RStructural constituent of ribosomeC4R7L9Putative uncharacterized proteinTom5p (39%)MProtein targeting to mitochondrionC4QVS9Plasma membrane H ⁺ -ATPase, pumps protons out of the cellPma1p (86%)PMProton transport, regulation of pHF2QT41Alcohol dehydrogenase class-3Yim1p (30%)LD/C/MResponse to DNA damage stimulusF2QYU4AN1-type zinc finger protein YNL155WYnl155wp (30%)C/NUnknownC4R4C3Mitochondrial matrix ATPaseSsc1p (82%)MProtein import into mitochondrial matrix, protein foldingC4QXL9GTPase, similar to Ypt51p and Ypt53p and to mammalian rab5Vps21p (54%)EEndocytosis, protein targeting to vacuole matrix attractionC4QWQ5Prenyltransferase, required for cell viability for exocytosisNus1p (47%)LD/ERProtein glycosylationC4R3N7Secretory vesicle-associated Rab GTPase essential for exocytosisNus1p (49%)ER/G/MER to Golgi vesicle-mediated transport	C4R7R7	Protein with similarity to oxidoreductases.	Env9p (48%)	LD	Vacuolar protein processing, vacuole organization
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for exocytosis	C4R3N7	Secretory vesicle-associated Rab GTPase essential	Ypt1p (49%)	ER/G/M	ER to Golgi vesicle-mediated transport
		for exocytosis			-

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