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Energy Storage in Yeast: Regulation and Competition with Ethanol Production

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Abstract Mechanisms that may regulate the storage of energy as triacylglycerol in *Saccharomyces cerevisiae* were examined. First, the kinetics of Dga1p, which mediates the majority of diacylglycerol esterification, the lone committed step in triacylglycerol synthesis, was measured in vitro. With an apparent K_m of 17.0 μM, Dga1p has higher affinity for oleoyl-CoA than the only *S. cerevisiae* acyltransferase previously kinetically characterized, Lpt1p. Lpt1p is a 1-acylglycerol-3-phosphate *O*-acyltransferase that produces phosphatidate, a precursor to diacylglycerol. Therefore, limiting triacylglycerol synthesis to situations of elevated acyl-CoA concentration is unlikely. However, Dga1p's apparent V_{max} of 5.8 nmol/min/mg was 20 times lower than Lpt1p's. This supports Dga1p being rate limiting for TAG synthesis. Dga1p activity was not activated or inhibited when seven different molecules (e.g., ATP) which reflect cellular energy status were provided at physiological concentrations. Thus, allosteric regulation was not found. Coordination between triacylglycerol and glycogen synthesis was also tested. Yeast genetically deficient in triacylglycerol synthesis did not store more energy in glycogen and vice versa. Lastly, we tested whether genetically limiting energy storage in triacylglycerol, glycogen, steryl esters, or combinations of these will increase ethanol production efficiency. In nutrient-rich media containing 5 % glucose, solely limiting glycogen synthesis had the greatest affect, increasing ethanol production efficiency by 12 %. Since limiting glycogen synthesis only had a modest effect on growth in media containing 10 % ethanol, such genetic manipulation may improve commercial ethanol production.

Introduction

Yeast such as *Saccharomyces cerevisiae* primarily store energy when nutrients, particularly nitrogen, are in short supply. When *S. cerevisiae* was shifted from nutrient-rich media to nutrient-poor sporulation media for 16 h, glycogen mass per cell increased nine fold [15]. Similarly, when *S. cerevisiae* was shifted to sporulation media for 24 h, relative triacylglycerol (TAG) mass increased 11-fold, while phospholipid (PL) mass only increased 2.5fold [13]. This storage precedes spore formation and provides a source of energy and structural components upon germination. Glycogen synthesis proceeds as glycogen synthase utilizes UDP-glucose to extend glycogen polymers. *S. cerevisiae* has two glycogen synthase isoforms, Gsy1p and Gsy2p. Combined deletion of these essentially eliminated glycogen production [10]. Nutrient availability affects the phosphorylation state of a variety of transcription factors [35]. These in turn influence the abundance of glycogen synthases [9] and thus regulate glycogen synthesis.

In contrast, the mechanisms that regulate TAG synthesis are less well understood. Indirect evidence suggests that

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phosphorylation of Gat2p, one of the two isoforms of glycerol-3-phosphate 1-*O*-acyltransferase (GPAT, Fig. 1), inhibits it [7]. Since GPAT is the initial reaction in the pathway for TAG and PL synthesis, its activity will likely coordinately influence the abundance of both products. However, preparation for sporulation requires much more TAG than PL [13]. Phosphatidate phosphatase (PAP, Fig. 1) is another enzyme whose activity influences TAG synthesis. Pah1p is one of four PAP isoforms in *S. cerevisiae* and the one whose activity seems to be most important regarding TAG and PL synthesis [21]. Pah1p is inhibited by phosphorylation [21] during exponential growth. In stationary growth, *PAH1* transcription is increased and proteasomal degradation of Pah1p is increased [25]. This creates a unique situation in stationary phase where Pah1p abundance is low and its activity is high. Regulation of Pah1p is somewhat specific for TAG synthesis since its product, diacylglycerol (DAG), while a precursor to both PL and TAG, is mainly used for TAG synthesis [26]. These studies leave open the possibility for additional mechanisms for TAG synthesis regulation.

Regulation of Dga1p and Lro1p, the only two enzymes dedicated to TAG synthesis (Fig. 1) and obvious candidates for regulating TAG synthesis, has not been characterized. Dga1p is an acyl-CoA-dependent diacylglycerol *O*-acyltransferase (DGAT, EC 2.3.1.20) [22, 32]. Lro1p is a phospholipid:diacylglycerol acyltransferase (PDAT) [23]. Compound deletion of *DGA1* and *LRO1* reduced TAG synthesis by over 95 % [22]. Further deletion of *ARE1* and *ARE2*, which encode for enzymes that primarily esterify sterols [36], completely eliminated TAG synthesis [23, 32]. Deletion of *LRO1* reduced DAG esterification by 75 % in log phase yeast but had no effect in stationary phase [22]. Therefore, Dga1p seems to be the major DAG esterifying enzyme in stationary phase when TAG production is particularly active. This makes Dga1p a good candidate for regulating TAG synthesis.

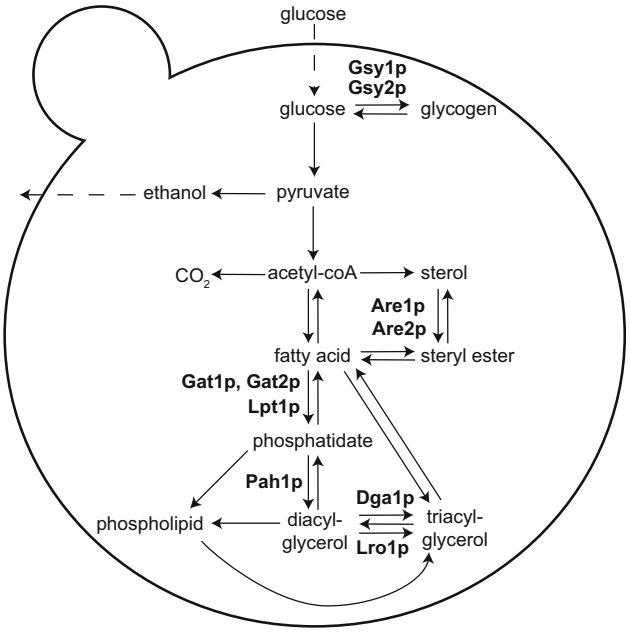

Fig. 1 Schematic of *S.cerevisiae* catabolism of glucose and anabolism of glycogen and major lipids For simplicity, not all intermediates and substrates are shown and some arrows correspond to more than one reaction. Enzymes of interest are in *bold*

In contrast to stationary phase, in nutrient-rich conditions, the production of glycogen and TAG is superseded by catabolism that yields ethanol (Fig. 1). Commercial ethanol fuel production, primarily involving the catabolism of hexoses by *S. cerevisiae*, yielded 25.7 billion gallons in 2015 worldwide (U.S. Dept. of Energy). Even though the efficiency for ethanol production has approached the theoretical maximum of 51 % in some growth conditions such as 20 % (w/v) glucose in soy flour media [5], efforts have continued to engineer yeast to be more efficient [37], more tolerant to high concentrations of ethanol [2, 16], and better able to use a variety of carbon sources [38].

How cells allocate nutrients and thus allocate energy is central to survival and commercial utilization. Due to being able to readily manipulate its genome, *S. cerevisiae* was chosen to better understand how TAG synthesis is regulated and determine if limiting energy storage will promote ethanol production.

Materials and Methods

Materials

Chemicals were mainly obtained from Sigma, Fisher, Sunrise Science Products (yeast media) and Avanti Polar Lipids (lipids). [1-^{14}C]oleoyl-CoA and [9,10-^{3}H(N)]oleate was from Perkin Elmer.

Yeast Strains and Culturing

Molecular biology and genetic procedures were performed according to conventional protocols [4]. All *S. cerevisiae* strains used were W303 haploids *(ade2-1, can1-1, trp1-1, ura3-1, his3-11, 15, leu2-3, 112)* [34]. Strains harboring compound deletion mutations of *ARE1*, *ARE2*, *DGA1*, and *LRO1* were generated previously [22, 36]. Strains harboring single-gene deletion mutations for *GSY1* and *GSY2* were generated by transforming normal haploids, W303-1A or 1B, with PCR-derived products containing 50 bp of gene-specific sequence flanking the *Kluyveromyces lactis URA3* sequence [29]. Genotypes were confirmed by PCR using internal and flanking oligonucleotides. Compound deletion strains were generated by mating single- or multiple-gene deletion strains, sporulation of the heterozygous diploids,

dissection of tetrads, and genotyping by PCR. Online Resource 1 lists the genotypes of strains used in this study. Yeast were routinely cultured in YEP (2 % (w/v) peptone, 1 % (w/v) yeast extract) with 2 % (w/v) glucose (YPD) at 30 °C. Specialized media included YEP with 5 % (w/v) glucose and 0.5 mM magnesium sulfate and YEP agar with 2 % (w/v) glucose and 6, 8, or 10 % (v/v) ethanol.

Microsome Isolation and In Vitro Diacylglycerol Acyltransferase Assays

Yeast were grown in YPD into early stationary phase ($Abs_{660} = 1.0$) and harvested. Cells were washed, lysed, and microsomes prepared by sedimentation at 100,000 $\times g$ as described [23]. DGAT activity was measured by incubating 40 μg of microsomal proteins, 160 μM dioleoylglycerol (in ethanol), 2.5–45 μM [1-^{14}C]oleoyl-CoA (20,000 dpm/nmol), 175 mM Tris, pH 7.8, 10 mM sodium acetate, 2 mg/ml bovine serum albumin (essentially fatty acid free), and 8 mM $MgCl_2$ in 200 μl at 23 °C for 5 min. Pilot experiments showed linearity for at least 10 min (data not shown). After the reactions were stopped, the lipids were extracted, resolved by thin-layer chromatography, and TAG quantified by scintillation counting.

Glycogen Concentration Assay

Yeast were grown in YPD into various phases (Abs_{660} equivalents = 1.2–3.6) and harvested. As described in [24], glycogen concentration was determined by lysing yeast in 0.25 M sodium carbonate, neutralization with acetic acid and sodium acetate, hydrolysis of glycogen into glucose monomers by amyloglucosidase, and a glucose oxidase-based colorimetric assay.

In Vivo, TAG Synthesis Assay

As described in [22], yeast were cultured in YPD at 30 °C into log phase. 4 ml of cells were mixed with 2 μM of [^{3}H]oleate (2.63 Ci/mmol) at 30 °C for 30 min. Cells were washed, cell walls digested with lyticase, and lipids extracted with hexane: isopropanol (2:1) and 2 M potassium chloride:methanol (4:1). Major lipid classes were resolved by thin-layer chromatography and quantified by scintillation counting. TAG abundance was expressed as a percent of tritium incorporated into all lipids.

Media Glucose Concentration Assay

Glucose concentration was determined by incubating either glucose standards or conditioned media, 0.1 M sodium phosphate, pH 7.0, 0.025 units of glucose oxidase, 1.25 units of horseradish peroxidase, and 1.67 mg 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) in 1 ml at room temperature for 30 min followed by measuring Abs_{735}.

Media Ethanol Concentration Assay

Ethanol concentration was determined by incubating either ethanol standards or conditioned media, 0.1 M potassium phosphate pH 7.5, 0.02 units of alcohol oxidase, 1.5 units of horse radish peroxidase, and 1 mg ABTS in 1 ml at room temperature for 30 min followed by measuring Abs_{735}.

Statistical Analysis

All percentile (p) data were transformed using a logit function, $\text{logit} = \ln(p/(1-p))$ [1], prior to analysis. Univariate comparisons were performed using a Student's t test. SigmaPlot software generated Hill plots to determine K_m and V_{max} values from kinetic data.

Ethanol Tolerance Assays

Yeast were cultured in YPD into log phase. After customized serial dilution, 4000, 400, 40 and 4 cells of each strain were sequentially spotted onto YPD agar with 6, 8, or 10 % (v/v) ethanol. Plates were incubated at 30 °C for 4 (6, 8 %) or 5 days (10 %) prior to photography. Growth was also measured in liquid YPD with or without 10 % (v/v) ethanol. Two strains per genotype were grown in 10 ml YPD into log phase on 2 separate days. From each culture, 2.6×10^7 cells were harvested by centrifugation, suspended in 4 ml of the respective media, and cultured at 30 °C for 6 h. Cell density, as Abs_{660}, was measured at 0, 3, and 6 h time points. Doubling time = $(\text{Time}_2 - \text{Time}_1) \times \log 2/\log (\text{Abs}_2/\text{Abs}_1)$.

Results

We hypothesized that mechanisms exist to regulate TAG synthesis independently from PL synthesis. This would separate the control of energy storage from the control of membrane component production. *S. cerevisiae* was chosen as the model system due to its genetic tractability and its use in bioengineering. Diacylglycerol O-acyltransferase (DGAT) was chosen as a candidate regulated reaction since it is unique to TAG synthesis. Dga1p primarily mediates this activity in *S. cerevisiae* (Fig. 1) [22, 32]. *DGA1* is a member of the DGAT2 gene family [17] that has homologs throughout the fungal, plant, and animal kingdoms [6] so the findings may have broad applicability.

One possible mechanism for limiting TAG synthesis to situations when acyl-CoA substrates are elevated is by Dga1p having low affinity for acyl-CoA. To test this, DGAT assays were performed using microsomes isolated from a *S. cerevisiae* strain with the three genes, *LRO1*, *ARE1*, and *ARE2* that encode for other DAG esterifying enzymes deleted. Analyzing the relationship between velocity and oleoyl-CoA concentration (Fig. 2) allowed the determination of the kinetic parameters apparent K_m and apparent V_{max} for Dga1p. These values are "apparent" since the use of hydrophobic substrates involves additional steps such as incorporation into membranes and lateral migration. Also, the sigmoidal relationship between oleoyl-CoA and DGAT activity prevented true Michaelis–Menten parameters from being calculated. Transforming Dga1p activity without ATP (Fig. 2) to a Hill Plot determined that the apparent K_m was 17.0 ± 5.6 μM. This is almost threefold lower than the apparent K_m for oleoyl-CoA use by Lpt1p, the only other yeast acyltransferase to be kinetically characterized [14, 28]. Lpt1p esterifies lysophosphatidate to make phosphatidate, a precursor to DAG (Fig. 1). Therefore, Lpt1p is more likely to be substrate-limited than Dga1. Since the cellular concentration of acyl-CoA in yeast has not been established, the extent to which Dga1 is substrate-limited cannot be established. Even so, these data are not consistent with the DGAT reaction being a "spill over" reaction that only occurs at high acyl-CoA concentrations.

Kinetic analysis of Dga1 also determined that the apparent V_{max} was 5.8 ± 2 nmol/min/mg. This is 20-fold less than the apparent V_{max} of Lpt1p [14, 28]. Dga1p's relatively low apparent V_{max} may be caused by limited solubility of DAG. Dga1p may also be a high-affinity, low capacity enzyme. In that case, DGAT may be an enzyme-limited reaction that is likely regulated by altering protein abundance. The relatively low velocity of Dga1p also suggests it may be rate limiting for TAG synthesis.

The sigmoidal relationship observed in (Fig. 2) suggests that Dga1p binds substrate cooperatively and may be allosterically regulated by the binding of metabolites [30]. To test this, the kinetics of Dga1 was determined in the presence of four metabolites whose elevated concentration indicates energy surplus. With the physiological concentration in cells replete with energy in parentheses, these are ATP (5 mM), NADH (0.8 mM), acetyl-CoA (0.2 mM), and malonyl-CoA (0.1 mM). Similarly, assays were performed with physiological concentrations of molecules whose elevated concentration indicates energy deficit: AMP (0.1 and 1 mM), ADP (0.1 mM), and NAD^+ (0.8 mM). As shown for ATP in (Fig. 2), none of the potential allosteric regulators significantly affected the affinity (K_m) or capacity (V_{max}) of Dga1p. Therefore, no evidence for allosteric regulation was observed.

Previous work showed deletion of a glycogen phosphorylase, *GPH1*, increased glycogen accumulation, and reduced TAG and steryl ester storage in *S. cerevisiae* [11]. To further examine the coordination of energy storage, yeast deficient in glycogen synthesis or TAG synthesis were assayed for TAG synthesis (Fig. 3) and glycogen abundance (Fig. 4), respectively. In both cases, there was no evidence for a compensatory increase in one form of energy storage if the formation of the other was compromised.

The relationship between energy storage and ethanol production was investigated next. We hypothesized that reducing energy storage will cause a higher percentage of carbons that enter cells as glucose to exit as ethanol (Fig. 1). Accordingly, yeast strains were generated that had

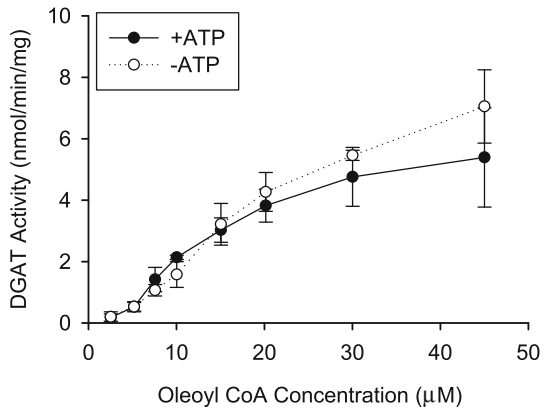

Fig. 2 Kinetic analysis of the diacylglycerol O-acyltransferase, activity mediated by Dga1p was measured using microsomes isolated from *lro1Δare1Δare2Δ* yeast. 40 μg of microsomes were incubated with 160 μM DAG and a concentration series of [1-^{14}C]oleoyl-CoA for 5 min at 23 °C. TAG was resolved by TLC and counted (*open circle*). Parallel assays included 5 mM ATP to examine allosteric regulation (*closed circle*). $n = 3$

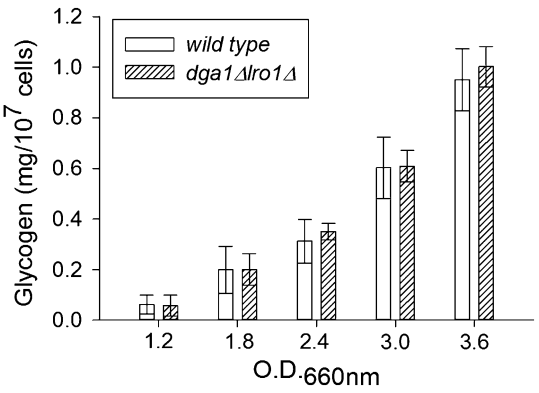

Fig. 3 Glycogen abundance in strains lacking triacylglycerol synthesis Wild type or *dga1Δlro1Δ* strains were cultured in YPD overnight until the cultures reached the indicated Abs_{660}. Cells were harvested, lysed, and glycogen mass measured as described in "Materials and Methods" section. $n = 3$

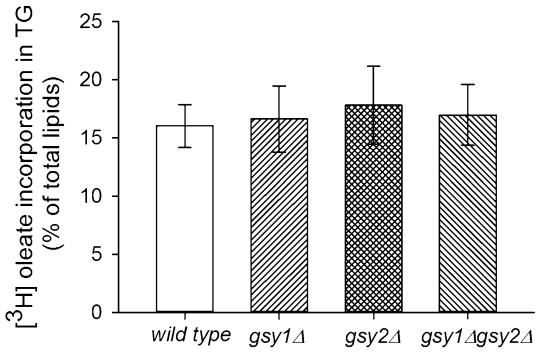

Fig. 4 Triacylglycerol synthesis in strains lacking glycogen synthesis Strains of the indicated genotypes were cultured in YPD overnight until the cultures reached $Abs_{660} = 0.4–0.8$. 4 ml of cells were incubated with 2 μM [^{3}H]oleate for 30 min, harvested, and lysed. Lipids were extracted, resolved by TLC, and counted. TAG abundance is reported as the percent of all lipids in the sample. $n = 3$

genotypes that caused dramatically reduced production of glycogen, TAG, and/or ergosterol esters (Online Resource 1). Ergosterol esters are not an interchangeable form of energy storage. However, since the synthesis of ergosterol from acetyl-CoA is energetically expensive, the formation of ergosterol esters provides an indirect storage of cellular energy. The genetic background was W303. It is commonly used for genetic studies but not for industrial ethanol production. YEP with 5 % glucose was chosen as the media to provide a nutrient-rich environment without the osmotic challenge of media with 20 % glucose used in some commercial settings. At 3-h intervals over a 12-h time course, cell abundance, media glucose concentration, and media ethanol concentration were measured (Fig. 5).

Combined deletion of *GSY1* and *GSY2* caused unique phenotypes. Cell abundance reached higher levels than the wild-type and other mutant strains (Fig. 5a). While glucose consumption proceeded similar to wild type (Fig. 5b), *gsy1Δgsy2Δ* had distinctively more ethanol production, especially after 6 and 9 h (Fig. 5c). The average ethanol production efficiency (mass of ethanol produced/mass of glucose consumed) for *gsy1Δgsy2Δ* strains at all four time points was 41.8 %. This was 12 % higher than the efficiency of wild-type strains, 37.3 % ($p = 0.07$). None of the other mutant genotypes caused a significant increase in ethanol production efficiency.

In addition to improved ethanol production efficiency, yeast with improved ethanol tolerance may benefit commercial ethanol production. Past studies have shown that lipid composition of cell membranes influences tolerance to environmental ethanol [12]. Reducing ergosterol storage may increase the abundance of free ergosterol in membranes and reducing TAG synthesis may facilitate PL synthesis and/or change the acyl chain composition of PL. To assess the effect of the genotypes on ethanol tolerance,

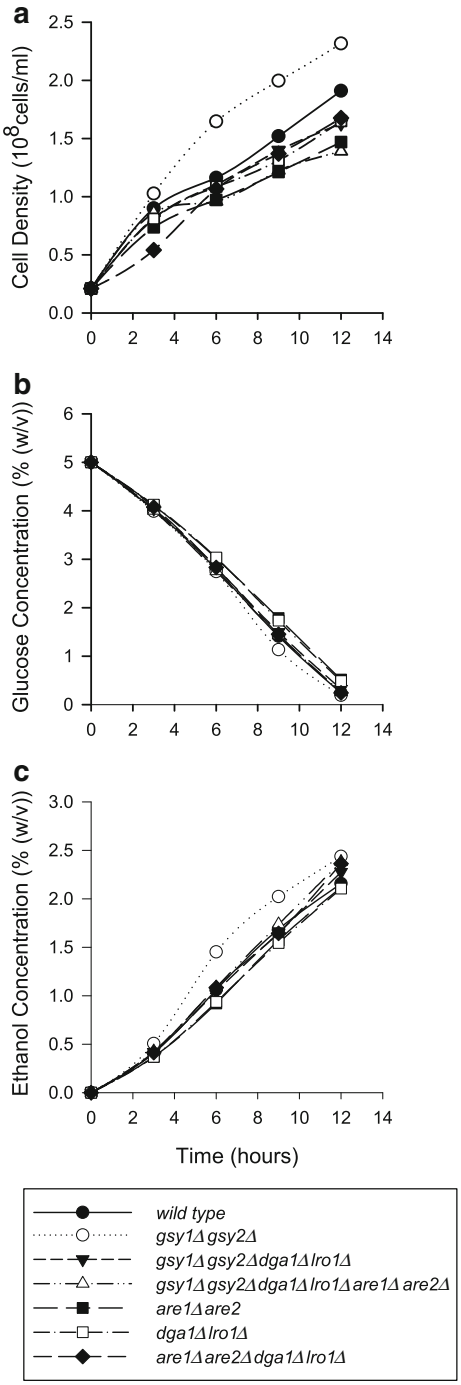

Fig. 5 Growth, glucose consumption, and ethanol production of yeast with energy storage deficiency *S. cerevisiae* lacking glycogen (*gsy1Δgsy2Δ*), triacylglycerol (*dga1Δlro1Δ*), steryl esters (*are1-Δare2Δ*), a combination of those, or wild type were cultured in 80 ml YPD into log phase, collected, and provided with 30 ml fresh YEP with 5 % (w/v) glucose and 0.5 mM $MgSO_4$ for a 12-h time course. 700 μl aliquots were harvested every 3 h. **a** Cell density was monitored by measuring Abs_{600}. **b** Media glucose concentration was monitored by a glucose oxidase based, in vitro assay. **c** Media ethanol concentration was monitored by an alcohol oxidase based in vitro assay. Error bars were not included to ease data point resolution. $n = 3$

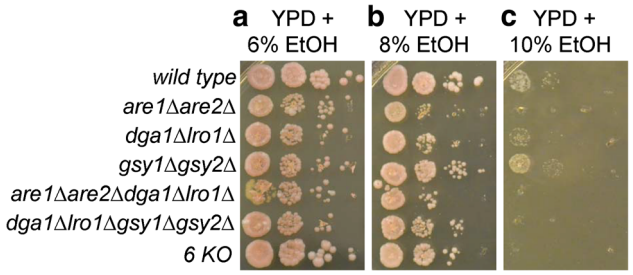

Fig. 6 Ethanol tolerance of yeast with energy storage deficiency Strains of indicated genotypes were cultured in YPD into log phase. Cell populations were diluted so that spotting onto the indicated agar plates delivered 4000, 400, 40, and 4 cells to sequential spots. Culturing occurred for 4 (6, 8 % ethanol) or 5 days (10 % ethanol). 6 KO is *1Δare2Δdga1Δlro1Δgsy1Δgsy2Δ*

serial dilutions of the respective strains were grown on solid media containing increasing amounts of ethanol. In 6 % ethanol, no phenotypes were observed (Fig. 6a). Interestingly, in 8 % ethanol (Fig. 6b), all of the strains which included *gsy1Δgsy2Δ* in the genotype grew as wild type, while all the strains with diminished neutral lipid production only: *are1Δare2Δ*, *dga1Δlro1Δ,* and *are1Δare2Δdga1Δ lro1Δ*, showed fewer cells at the second dilution and almost no cells at the third dilution. This is consistent with limiting neutral lipid synthesis increasing ethanol sensitivity and this sensitivity being counteracted by the absence of glycogen. Perhaps the reduction in autophagy previously observed in *dga1Δlro1Δ* and *are1Δare2Δ* strains caused the increased ethanol sensitivity [33]. In 10 % ethanol, an amount that may be generated in commercial ethanol production, only *gsy1Δgsy2Δ* strains grew as well as wild type on solid media.

To quantify the effect of ethanol on growth, the doubling time of two strains of each genotype was determined in log phase in YPD or YPD with 10 % ethanol (Table 1). While none of the mutations slowed growth in YPD, all of the mutations showed a significant increase in doubling time in the presence of 10 % ethanol. Even though long-term ethanol exposure on solid media and short-term exposure in liquid media may provide distinct challenges, the genotypes *dga1Δlro1Δ* and *gsy1Δgsy2Δ* that had the smallest increases in doubling time also showed visible growth on YPD agar with 10 % ethanol. In liquid media, removing TG storage showed less of an effect than on solid media.

Discussion

Determining the reactions in TAG synthesis that are regulated and the mechanisms of the regulation will provide a better understanding of energy balance. Knowledge of this regulation may also inform the bioengineering of yeast to more efficiently produce TAG and other acyl-containing compounds for commercial purposes. Strategic manipulation of pathways is also improved when the rate limiting steps, which are often regulated, are identified. While the regulatory mechanisms we hypothesized for the main yeast DGAT, Dga1p, were not confirmed by experimentation, the data suggest Dga1p is among the rate limiting steps in TAG synthesis. The apparent V_{max} of Dga1p, 5.8 nmol/min/mg, is identical to the GPAT activity when yeast microsomes were supplied with 40 μM oleoyl-CoA [3]. That GPAT activity may not be maximal. The lysophosphatidate generated by the GPATs can be utilized by two AGPATs, Lpt1p [22, 28] and Slc1p [20]. Lpt1p has a V_{max} of 125 nmol/min/mg [14] and Slc1p had a velocity of 20 nmol/min/mg with 50 μM oleoyl-CoA [28]. Microsomal activity of PAP, which converts phosphatidate to DAG, supplied with 2 mM phosphatidate was 10 nmol/min/mg [19]. While the heterogeneous conditions of the various in vitro assays prevent definitive comparisons, the activity of Dga1 is on the low end of the reported activities for enzymes in the TAG synthetic pathway. Dga1p being rate limiting agrees with the finding that replacing the

Table 1 Doubling times of normal and storage-deficient strains in log phase in YPD and YPD + 10 % ethanol

Genotype	Doubling time in YPD (min)	Doubling time in YPD+ 10 % ethanol (min)
Wild type	122 ± 7	574 ± 25
are1Δare2Δ	123 ± 10	896 ± 181*
dga1Δlro1Δ	112 ± 5	687 ± 71*
gsy1Δgsy2Δ	121 ± 7	782 ± 60*
are1Δare2Δdga1Δlro1Δ	117 ± 3	849 ± 195*
dga1Δlro1Δgsy1Δgsy2Δ	136 ± 30	865 ± 57*
6 *KO*	126 ± 6	911 ± 181*

Standard deviations are included

* Significantly different ($p < 0.05$) than wild type in the same media. $n = 4$

endogenous *DGA1* promoter with a more active, *TEF1*, promoter increased TAG production 2.5-fold [31].

Increasing ethanol production efficiency was observed when *GSY1* and *GSY2* were deleted. One mechanistic explanation is that in the absence of glycogen synthesis, more glucose 6-phosphate is available for glycolysis and subsequent ethanol production. The concomitant increase in ATP production may facilitate anabolic processes involving nonmonosaccharide nutrients that allow *gsy1Δgsy2Δ* strains to reach a higher cell density prior to plateauing. Preventing glycogen synthesis may also improve metabolic efficiency by eliminating the energy loss incurred by synthesizing the glycogen polymer and later hydrolyzing it. While the same efficiency may be derived by limiting TAG synthesis, the small amount of acetyl-CoA generated when glucose is plentiful may limit the scale of this benefit.

The 41.8 % ethanol production efficiency in *gsy1Δgys2Δ* is distinctly lower than the theoretical yield of 51 %. The other 49 % of glucose mass is unavoidably converted to carbon dioxide during fermentation. The theoretical yield has been very nearly achieved by immobilizing *S. cerevisiae* strain SC-20 on beads and providing 250 or 300 g/L cane sugar and soya flour [5]. Therefore, the current study has not necessarily generated an improved strain for commercial ethanol production. However, a genetic strategy for improving the efficiency of ethanol production in commercially utilized strains is provided. Whether deleting *GSY1* and *GYS2* will reduce yeast viability upon rehydration, as might be suggested by the observation that overexpressing *GSY2* increases yeast productivity upon rehydration [27], remains to be determined. We did establish that removing glycogen synthesis leads to, at most, a modest increase in ethanol sensitivity depending on the assay utilized. In fact, in 8 % ethanol in solid media, the phenotype of increased sensitivity conferred by eliminating neutral lipid synthesis was reversed upon concomitant elimination of glycogen synthesis. Eliminating glycogen synthesis may allow more UDP-glucose to be allocated for trehalose synthesis. Trehalose provides relief from cell stresses [8] and promotes ethanol tolerance [18].

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Compliance with Ethical Standards

Conflict of Interest Silpha Jain contributed to this manuscript, experiments, and writing, while she was a graduate student at Drexel University. She is now employed by Trac Services, a pharmaceutical regulatory consulting company as a senior regulatory affairs executive. No pharmaceuticals were used in this study. There are no other potential conflicts of interest to report.

Research Involving Human and Animal Rights No human or animal subjects were used in these studies.

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