

## Research Article

# A Transcriptional Regulatory System of the *S. cerevisiae* *OLE1* Gene Responds to Fatty Acid Species and Intracellular Amount, and not Simply Membrane Status

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We examined the effects of unsaturated fatty acid (UFA) species and their concentration on the expression of *OLE1*, which encodes the stearyl CoA desaturase, in *Saccharomyces cerevisiae*. We controlled the amount of UFA taken up by the cell by varying the concentration of tergitol in the medium. When cultured with 1 mM fatty acid in 0.1% tergitol, cells took up much more fatty acid than when cultured with the same concentration of fatty acid at 1% tergitol, although the amount incorporated was dependent on UFA species. For each fatty acid tested, we found that the higher uptake (0.1% tergitol condition) had a stronger impact on *OLE1* regulation. A principal product of the desaturase 16:1 $\Delta$ 9, and the nonnative UFA 18:2 $\Delta$ 9,12, most strongly repressed the reporter construct *OLE1-lacZ* transcription, while the other major product of the desaturase, 18:1 $\Delta$ 9, and the nonnative UFA 17:1 $\Delta$ 10 caused a more diminished response. Based on these results, our initial hypothesis was that *OLE1* was regulated in response to membrane fluidity; however, subsequent work does not support that idea; we have found that conditions that affect membrane fluidity such as growth temperature and growth with saturated or *trans* fatty acid supplementation, do not regulate *OLE1* in the direction predicted by fluidity changes. We conclude that at least one signal that regulates *OLE1* transcriptional expression is most likely based on the fatty acids themselves.

## 1. Introduction

In the yeast *Saccharomyces cerevisiae*, there is a single fatty acid desaturase encoded by the *OLE1* gene [1, 2]. The stearyl-CoA desaturase, which functions as a homodimer [3], introduces a *cis* double bond between carbons 9 and 10 of the acyl chain. Its primary products *in vivo* are palmitoleic (16:1 $\Delta$ 9) ( $x:y\Delta n$ , fatty acids containing  $x$  carbon atoms and  $y$  *cis* double bonds located at position  $n$  from the carboxyl end; UFA, unsaturated fatty acid) and oleic acid (18:1 $\Delta$ 9) [4]. There is accumulating evidence that aberrant expression of the stearyl-CoA desaturase is also involved in disease; in mammals, an anticancer gene ORCTL3 was identified that could cause apoptosis in certain transformed cells [5]. Later research identified the target of ORCTL3 as the stearyl-CoA desaturase [6]. And in yeast, overexpression of stearyl-CoA desaturase leads to invasive growth [7]. Recent investigations have identified a role of *ARV1* in fatty liver disease [8]. The yeast *arv1* mutants are defective in *OLE1* regulation, implying a possible connection

between fatty liver disease and desaturase expression. Recently, Vincent et al. have demonstrated the potential of the desaturase as a druggable target for treatment of neurodegenerative disorder such as Parkinson's disease [9].

*OLE1* expression is regulated at the level of transcription, RNA stability and protein activity (reviewed in [10, 11] and is known to be regulated by various growth conditions, including exogenous unsaturated fatty acids [12–14], cobalt and carbon source [15], ethanol concentration [16, 17], and oxygen [18]. While environmental conditions such as culture temperature have been investigated, these studies have generally found transient changes in expression with a small long term impact on fatty acid composition [19, 20]. The best characterization of these regulatory systems is the positive regulation of *OLE1* when cells are grown in the absence of products of the desaturase: UFAs containing a double bond between carbons 9 and 10. Previous work has determined that transcription of the *OLE1* gene is controlled, at least in part, by homologous transcription factors, encoded by the genes *MGA2* and *SPT23* [21]. When

there are abundant UFAs, the Mga2p and Spt23p transcription factors are tethered as inactive p120 forms in the endoplasmic reticulum membrane. But, when UFAs are limiting, these two proteins are proteolytically cleaved from their carboxy tails, and the soluble p90 N-termini are translocated into the nucleus, where they can activate transcription of the *OLE1* gene [21].

The processing of Mga2p and Spt23p into their active p90 forms occurs through a controlled, ubiquitin-regulated proteolysis by the proteasome in the endoplasmic reticulum. A complex of the proteins Cdc48p-Npl4p-Ufd1p is required for this activation of Mga2p and Spt23p [22–24]. In addition, the *UBX2* encoded protein has been shown to be important in the regulation of *OLE1* in response to exogenous fatty acids, acting as a bridging factor between the cytosolic Cdc48p-Npl1p-Ufd1p complex with the membrane associated ubiquitin ligase complex and Mga2p and Spt23p [25, 26]. In addition to its role in regulating *OLE1*, Mga2p has been implicated in regulation of at least one other gene, *ERG1*, which is involved in sterol biosynthesis [27]. Interesting work by Covino et al. has shown that when Mga2p forms a homodimer, the orientation of the transmembrane helices can vary based on the lipid environment. The alternative conformations are stabilized differentially, depending on whether the protein is found in a membrane rich or poor in 18:2 $\Delta$ 9,12. These alternative conformations result in differing abilities of Mga2p to be processed into the active p90 form, hence allowing Mga2p to act as a lipid sensor [28].

However, UFAs are diverse in structure; varying by chain length, presence or absence of double bonds in the acyl chain, number and location of double bonds and whether the bonds are *cis* or *trans* configuration. We investigated whether some of these distinctions resulted in different regulatory schemes to control *OLE1* expression. In this work, we look at the effect of concentration and UFA structure of four UFAs on controlling desaturase expression: the authentic products of the desaturase, palmitoleic acid (16:1 $\Delta$ 9) and oleic acid (18:1 $\Delta$ 9), and two nonnative products linoleic acid (18:2 $\Delta$ 9,12), a polyunsaturated fatty acid with two double bonds, between carbons 9–10 and 12–13, and the fatty acid *cis*-10-heptadecenoic acid (17:1 $\Delta$ 10), an odd chain length monounsaturated fatty acid which does not contain a 9–10 bond, but a 10–11 bond. These fatty acids were carefully chosen so that we could examine how the various structural and physical characteristics affect regulation. The UFA 18:2 $\Delta$ 9,12 shares the same chain length as one of the authentic products of the desaturase, having in common the  $\Delta$ 9–10 double bond as both products, and a similar melting temperature as palmitoleic acid. However, as 18:2 $\Delta$ 9,12 is a polyunsaturated fatty acid, it is quite different structurally. The UFA 17:1 $\Delta$ 10 does not have a  $\Delta$ 9–10, but its double bond is the same distance from the methyl end as 16:1 $\Delta$ 9's bond. This nonnative UFA shares a similar melting temperature as 18:1 $\Delta$ 9. In addition, we examined conditions that alter membrane fluidity to try and detect a consistent molecular response.

## 2. Methods

**2.1. Strains, Media, and Growth Conditions.** The yeast strain W303-1A (*MATa, trp1-1, his3-11,15, ade2-1, leu2-3,112, ura3-1*)

was used in this study. Standard methods for yeast growth and media were as described [29]. YNBt medium is the standard synthetic complete medium (0.67% yeast nitrogen base, 2% dextrose) with 0.1% or 1% tertigol NP-40 (Sigma) as noted, supplemented with appropriate amino acids, uracil and adenine. Fatty acids were prepared as stock solutions at 50 or 100 mM concentration in ethanol and supplemented to YNBt media as noted. YNBt media without fatty acids contained 1% ethanol.

Yeast transformations were performed via the lithium acetate procedure [30] using reagents from Takara Bio. For routine propagation of plasmids, *E. coli* NEB5 Alpha (New England Biolabs) were used, grown in LB medium supplemented with ampicillin. Standard procedures for cloning, growth and selection of bacteria were followed [31]. The plasmid p62 has placed *lacZ* under control of a 1015-bp *HindIII/SalI* fragment of the *OLE1* gene, starting from base –934 relative to the start codon, and including the first 27 codons of the desaturase placed in frame with *lacZ* [32].

**2.2. Fatty Acid Analysis.** To analyze total fatty acids, cells were cultured at 30°C in 200 ml YNBt overnight to a maximum density of  $1-2 \times 10^7$  cells/ml. Fatty acids in ethanol (or ethanol alone for control) were added to cultures and incubation continued for four hours. Cells were harvested by centrifugation, washed with sterile water, and lipids were saponified and converted to methyl esters by a modified Bligh–Dyer method [33]. Samples of fatty acid methyl esters were analyzed by gas chromatography on a Hewlett–Packard model 6890 gas chromatograph using a 30 m HP-5 column. Instrument conditions were as described [34]. Identification of cellular fatty acid species was made by comparison of retention times to those of authentic fatty acid standards (Sigma).

**2.3. Beta-Galactosidase Assays.** Yeast bearing plasmid p62 were grown overnight in YNBt media with either 1% or 0.1% tertigol as noted. Cultures were inoculated into fresh media with either 1% ethanol (for control) or noted UFA and incubated at 30°C, shaking, for four hours before performing assays. Cells were harvested by centrifugation and  $\beta$ -galactosidase activity was measured using the permeabilized cell method [29]. Enzyme activity was determined using Equation (1).

$$\beta - \text{galactosidase activity} = \frac{A_{420}}{(A_{600})(\text{volume of culture tested})(\text{time})} \quad (1)$$

Assays for each construct were performed on at least five replicates. Activity was normalized to the ethanol controls (set at 100%).

**2.4. Fatty Acid Melting Point Determination.** 99% pure *cis*-10-heptadecenoic acid (Sigma) was loaded into three separate capillary tubes, melted, and centrifuged at about 2000 rpm for two minutes. The samples were then allowed to solidify. Using a Barnstead International model 1001D Mel-Temp melting point apparatus, a preliminary “fast” reading was taken (increase of 5–10°C/min) to determine the general range of the melting point of the sample. The instrument was then cooled to 10°C below estimated melting point, and a second sample was read at a slow rate (increase of 1°C/

min). The slow rate read was repeated. As a control, the melting temperature range of 99% pure *cis*-9 octadecenoic acid was determined through this same method and found to be 13.0–13.4°C, which matched the published value of 13.4°C well [35].

### 3. Results

**3.1. The Relative Intracellular Concentration of Exogenous UFAs Can Be Controlled by the Amount of Tergitol in the Medium.** In many experiments involving feeding of fatty acids to yeast cells, a compound with detergent properties, such as Tween or tergitol, is added to the growth medium to help keep the fatty acids solubilized and available to the cells. We hypothesized that the effective concentration of supplied fatty acids available to the cells may be controlled by the amount of detergent in the medium. We tested the effect of the amount of tergitol in the growth medium (at 0.1% and 1%) on the relative incorporation of a fed fatty acid, which was maintained at a concentration of 1 mM. As seen in Figures 1(a) and 1(b), the relative amount of fatty acid that was taken up was dependent on the particular species. But in each case, more of the fed fatty acid was found in the cell at 0.1% tergitol growth conditions than at 1% tergitol (Figure 1(c)).

By examining the fatty acid profiles, we determined that when cells are not supplemented with UFA, the desaturase is active, resulting in a large proportion of total fatty acids being unsaturated; 16:1 $\Delta$ 9 representing approximately 45% of total fatty acids, and 18:1 $\Delta$ 9 about 30%. When supplied 16:1 $\Delta$ 9 or 18:1 $\Delta$ 9, the fed fatty acid increases at the expense of the other. For example, when fed 16:1 $\Delta$ 9, we can clearly see that the amount of 18:1 $\Delta$ 9 produced by the desaturase has decreased substantially (Figures 1(a) and 1(b)). When we compare the 1% and 0.1% tergitol conditions in the case of 16:1 $\Delta$ 9 feeding, we observe 16:1 $\Delta$ 9 is a robust 85% of total fatty acids in the 0.1% tergitol condition, while approximately 55% of total fatty acids is 16:1 $\Delta$ 9 in the 1% tergitol culture conditions. Both increases are basically at the expense of 18:1 $\Delta$ 9, which decreased from the unfed condition of about 30% of total fatty acids to about 19% in the 1% tergitol condition and to 2% in the 0.1% tergitol condition. As it is impossible to distinguish under these conditions between native, endogenously produced 16:1 $\Delta$ 9 and fed 16:1 $\Delta$ 9, it is formally possible that the desaturase is still actively producing 16:1 $\Delta$ 9 when fed 16:1 $\Delta$ 9. However, the loss of production of 18:1 $\Delta$ 9 indicates that most likely the desaturase itself is not active. This inability to distinguish endogenous from exogenous is not a problem in the feeding of the nonnative fatty acids 17:1 $\Delta$ 10 and 18:2 $\Delta$ 9,12. When these fatty acids are fed, we see concomitant decreases in both 16:1 $\Delta$ 9 and 18:1 $\Delta$ 9, indicating clearly that the desaturase is not as active under these fed conditions. Interestingly, we observed that 18:2 $\Delta$ 9,12 is incorporated at a higher level than 17:1 $\Delta$ 10 under both culture conditions; about 21% 18:2 $\Delta$ 9,12 vs. 9% 17:1 $\Delta$ 10 in the 1% tergitol conditions, and 69% 18:2 $\Delta$ 9 vs. 35% 17:1 $\Delta$ 10 in the 0.1% tergitol.

**3.2. Regulation of Expression from the *OLE1* Promoter Is Dependent on Fatty Acid Species and Concentration.** In order

to quantify the effect of growth in UFA supplemented medium on *OLE1* transcriptional regulation, we examined the effects of the supplement on a reporter construct of the *OLE1* promoter controlling expression of *lacZ*. Growth in medium containing 16:1 $\Delta$ 9 or 18:2 $\Delta$ 9,12 resulted in a steep decline in activity of  $\beta$ -galactosidase as compared to unsupplemented condition, while there was an intermediate level of activity in 18:1 $\Delta$ 9 and 17:1 $\Delta$ 10 supplemented conditions (Figure 2).

When we examined the influence of a higher effective concentration of UFA availability in the 0.1% tergitol condition versus 1% tergitol, in general, we observed that increasing the concentration of UFA increases its effect on the *OLE1* promoter activity. However, 18:1 $\Delta$ 9 does not follow this trend, exhibiting similar decreases in expression despite differences in intracellular amounts.

**3.3. Conditions That Alter Membrane Fluidity Have Unexpected Effects on *OLE1* Expression.** Examining the evidence so far, we hypothesized that the sensor(s) that regulate *OLE1* are responding to membrane fluidity signals. This is consistent with the idea that when fed a UFA with a lower melting temperature, it would result in increased membrane fluidity, creating a need to decrease expression of *OLE1*. To test this hypothesis, we examined the effect of other conditions that would affect fluidity on *OLE1* expression; growth temperature and the presence of saturated or *trans* fatty acids.

Using the *OLE1-lacZ* reporter construct, wild type cells were grown in noted condition for four hours, after which  $\beta$ -galactosidase activity was measured. As seen in Figure 3(a), the effect of growth in saturated or *trans* $\Delta$ 9 UFAs was examined for effect on *OLE1* expression, and in Figure 3(b), the effect of temperature was examined on *OLE1* expression. One would predict that in rigidifying conditions, expression of the desaturase would be induced and in fluidizing conditions, expression would be repressed. However, rigidifying conditions of growth in saturated fatty acids resulted in no significant change in reporter activity, while growth in a *trans* UFA significantly decreased *OLE1* expression about 50% (Figure 3(a)). Indeed, growth at colder temperature (24°C) actually resulted in about 50% decrease in *OLE1* transcriptional activity as compared to the standard 30°C growth temperature condition (Figure 3(b)) while there was no significant difference in reporter activity between standard growth temperature of 30°C and 34°C (Tukey's range test,  $p < .05$ ).

Table 1 provides physical/structural properties of the fatty acids tested and summarizes the effects of the different fatty acids for their ability to regulate *OLE1* transcriptional activity as measured in the reporter assay.

### 4. Discussion

The uptake and use of fatty acids from the diet into the cell is a critical step, necessary for energy use and storage, and as precursors for complex lipid formation. However, as there is wide diversity in fatty acid structure, this may result in some fatty acids being ideal and some being less optimal for use under particular growth or environmental conditions. While other studies have investigated the role of exogenous fatty acids in

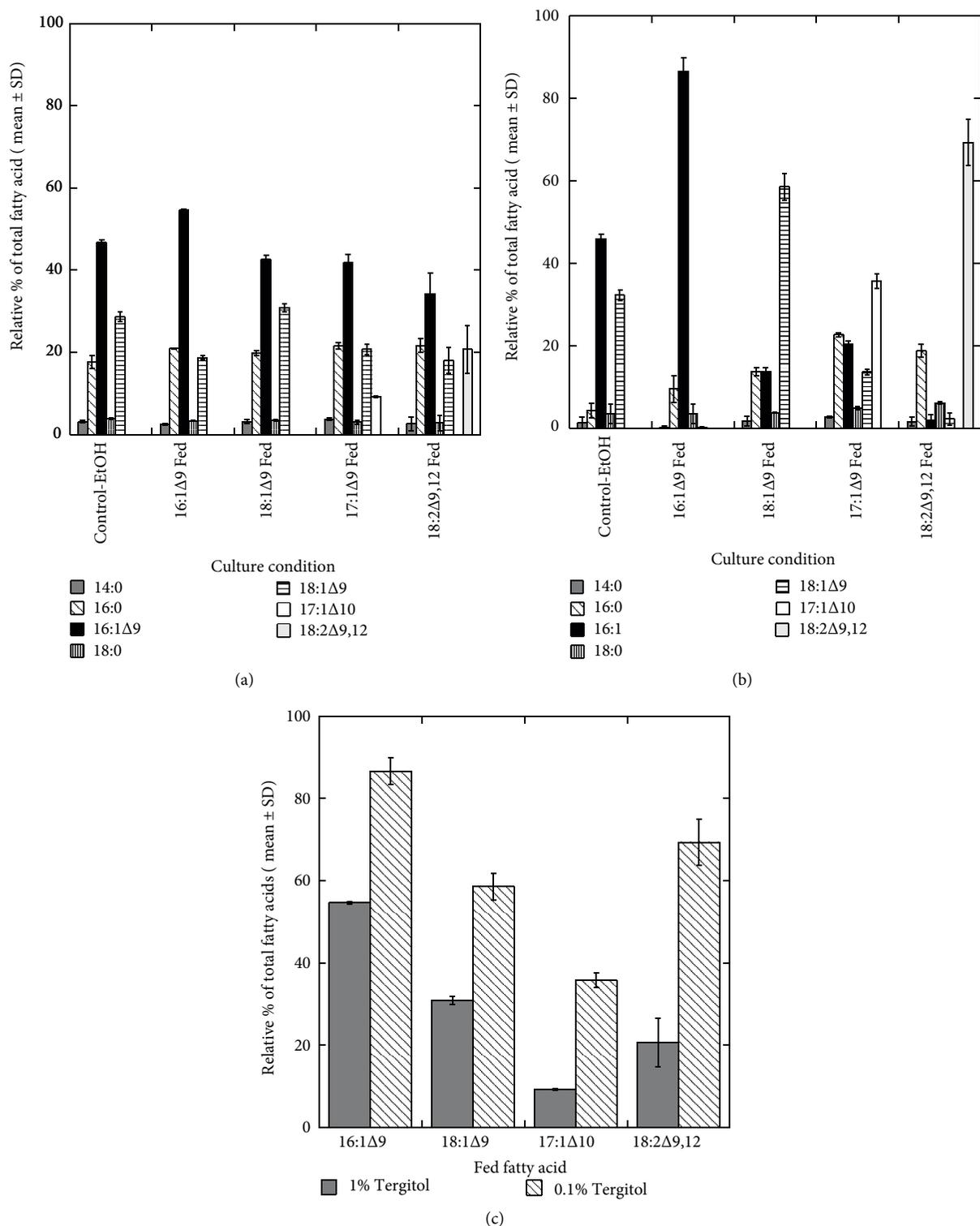


FIGURE 1: Fatty acid composition of cells grown  $\pm$  unsaturated fatty acids. Strain W303-1A was grown overnight and refreshed into YNBt medium with either 1% tergitol or 0.1% tergitol, containing 1 mM noted fatty acid, or ethanol for control, for four hours. Cells were harvested, fatty acid methyl esters prepared and analyzed by GC as described in Section 2. At least three replicates were analyzed for each growth condition. Fatty acid profiles of cells grown in the presence or absence of unsaturated fatty acid supplements in either (a) 1% tergitol or (b) 0.1% tergitol. (c) A comparison of the relative amount of fed UFA present in 1% and 0.1% tergitol culture conditions.

regulation of *OLE1*, we also varied the effective amount of UFA available to cells by varying the amount of tergitol. This allowed us to examine both the effect of intracellular concentration and

the structural differences on regulation. We found that cells fed 16:1Δ9 or 18:2Δ9,12 resulted in strongest regulation of desaturase expression. While it may not be surprising that supplying

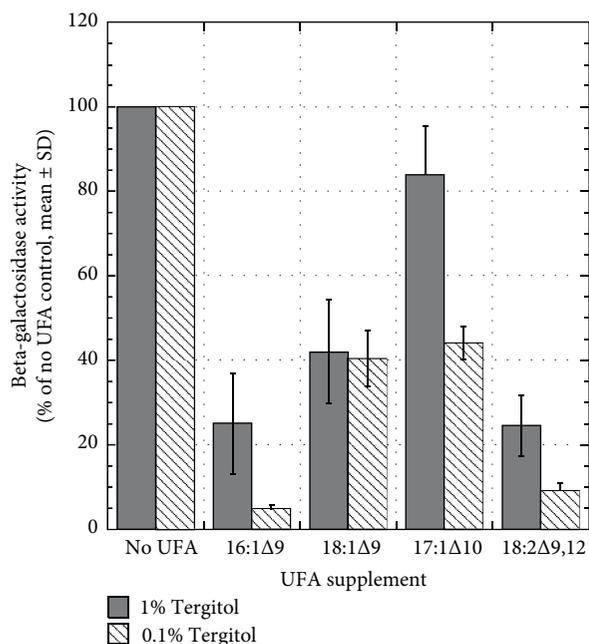
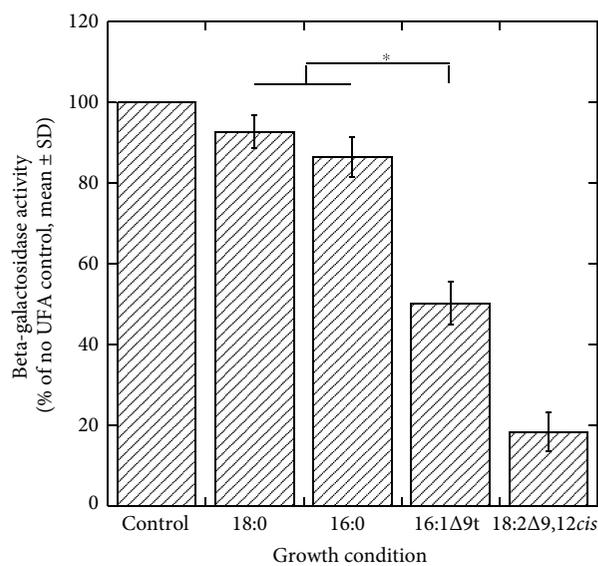


FIGURE 2: Effect of UFA species and tergitol concentration growth conditions on *OLE1* expression. Wild type strain W303-1A with plasmid p62 (*OLE1* promoter-*lacZ* reporter) was grown overnight, and refreshed into YNBt medium with either 1% tergitol or 0.1% tergitol, containing 1 mM noted fatty acid, or 1% ethanol for control, for four hours. Cells were harvested and  $\beta$ -galactosidase activity determined. Activity was normalized to ethanol control. At least 5 replicates were performed for each growth condition.

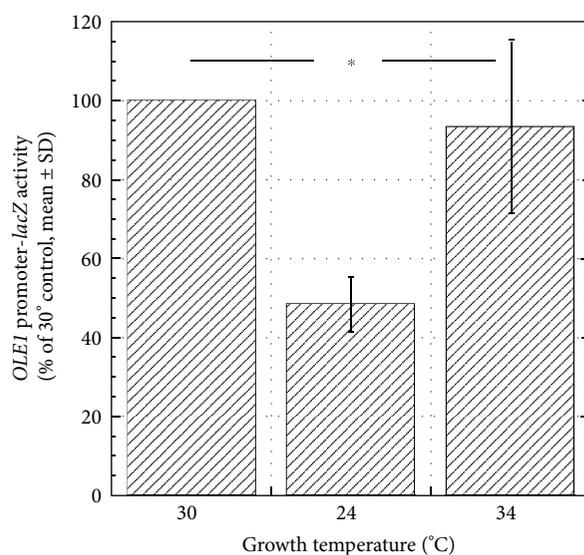
a product of the desaturase, 16:1Δ9, or even that a fatty acid that is somewhat similar to the monounsaturated product 18:1Δ9, such as 18:2Δ9,12 resulted in down regulation of the desaturase, it was unexpected that when fed 18:1Δ9, an authentic product of the desaturase, we observed an intermediate level of regulation. The presence of 17:1Δ10 led to a similar, tempered response as 18:1Δ9. Taken together, these results leave us to question “what is the actual signal for down regulation of expression of *OLE1*?”.

The regulation of *OLE1* is not simply by the products of the desaturase as evidenced by the strong repression by 18:2Δ9,12, or simply the presence of a Δ9 double bond, as 18:1Δ9 would then cause the same degree of effect as 16:1Δ9 or 18:2Δ9,12. When we examine the physical properties of these tested fatty acids (Table 1), they differ in mass, double bond number and location, but none of those properties seem to be correlated to the differences in regulation. We ruled out the possibility that that the sensor is measuring from the methyl end, as the double bond in both 17:1Δ10 and 16:1Δ9 are the same distance (7 carbons) from the methyl end. However, there is a correlation between melting temperatures and the degree of regulation (Table 1).

Excellent work by Covino et al. have uncovered that part of the signaling occurs through Mga2p's ability to sense the lipid environment through formation of different transmembrane helical domain interactions [28]. These different domain interactions then affect the susceptibility of Mga2p to be processed into the p90 active form. Given that finding and our results



(a)



(b)

FIGURE 3: Effect of membrane altering conditions on *OLE1* expression in wild type cells. Conditions thought to affect membrane fluidity were examined. Wild type W303-1A cells containing the *OLE1-lacZ* p62 reporter plasmid were grown overnight in YNBt (1% tergitol) and refreshed into noted condition for four hours. Cells were harvested and  $\beta$ -galactosidase activity determined. At least 5 replicates were performed for each growth condition. (a) Effect of growth on *OLE1* expression in minus (“control”) or plus saturated or *trans* fatty acids (18:2Δ9,12 *cis* is included as positive control). (b) Effect of temperature was examined on *OLE1* expression. 30°C is the normal growth temperature of yeast under laboratory conditions, and was used as the “control” condition. Asterisk indicates significant difference ( $p < 0.05$ ).

described so far, we then hypothesized that the regulatory system responsible for determining the need for expression of the desaturase is actually monitoring the membrane environment condition as opposed to presence or absence of particular molecules; in other words, the physical status of the membrane may be important to the transcriptional regulation of the *OLE1* gene.

TABLE 1: Correlation of physical and structural properties of the fatty acids used in this study with regulatory effect on *OLE1*. For most fatty acids, the melting points were as reported [35]. The melting point of *cis*-10-heptadecenoic acid was determined as described in Section 2.

Compound	Number of carbons	MW (daltons)	Number/type of double bonds in acyl chain	Position of bond(s) from carboxy end	Melting point (°C)	Ability to regulate <i>OLE1</i> expression (reporter gene assay)
<i>cis</i> -9 hexadecenoic acid (16:1Δ9)	16	254	1/ <i>cis</i>	9–10	−0.1°	+++
<i>cis</i> -9 octadecenoic acid (18:1Δ9)	18	282	1/ <i>cis</i>	9–10	13.4°	++
<i>cis,cis</i> -9,12 octadecadienoic acid (18:2Δ9,12)	18	280	2/ <i>cis</i>	9–10, 12-13	−12°	+++
<i>cis</i> -10 heptadecenoic acid (17:1Δ10)	17	268	1/ <i>cis</i>	10–11	15.7–15.9°	++
Hexadecanoic acid (16:0)	16	256	0	NA	145°	0
<i>trans</i> -9 hexadecenoic acid (16:1Δ9 <i>trans</i> )	16	254	1/ <i>trans</i>	9–10	33–34°	+
Octadecanoic acid (18:0)	18	284	0	NA	67°	0

However, when we examined the effect of conditions that we predict would have a rigidifying effect, lower growth temperature, or growth with saturated or *trans* fatty acids, we did not see the expected increase in *OLE1* gene expression. Additionally, growth at elevated temperatures, a condition that should increase membrane fluidity, did not decrease *OLE1* gene expression. While these culturing conditions may have had other effects on the cell that could impact *OLE1* expression, such as changing growth rate, they are consistent in their lack of support of a membrane fluidity sensor solely controlling *OLE1*. While it seems that membrane status is crucial, and is dependent on many considerations, including UFA content, our data indicate that it must not be simply the fluidity of the membrane, as conditions that affected fluidity did not fully impact regulation. These results imply that at least one part of the sensing system is not solely examining the physical status of the membrane and may be more specifically responding to a feature of the unsaturated fatty acid molecule. Further work is required to identify the nature of the sensing system.

### Data Availability

The chromatographic data to support the findings of this study are available from the corresponding author upon request.

### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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