APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1997, p. 910–915 Vol. 63, No. 3
0099-2240/97/$04.00+0

Effect of Aeration and Unsaturated Fatty Acids on Expression of the *Saccharomyces cerevisiae* Alcohol Acetyltransferase Gene

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Received 6 September 1996/Accepted 13 December 1996

The reduction of acetate ester synthesis by aeration and the addition of unsaturated fatty acids to the medium has been reported to be the result of the reduction in alcohol acetyltransferase (AATase) activity induced by inhibition of this enzyme. However, regulation of the AATase gene *ATF1* has not been reported. In this study, *ATF1* gene expression was studied by Northern analysis, and the results showed that the *ATF1* gene was repressed both by aeration and by unsaturated fatty acids. The results also showed that the reduction of AATase activity is closely related to the degree of repression of *ATF1* mRNA, which suggested that the gene repression is the primary means of reducing AATase activity in vivo. Using the *Escherichia coli lacZ* gene as a reporter gene, it was shown that a 150-bp fragment of the 5′ flanking sequence played a major role in the repression by aeration and unsaturated fatty acid addition.

The control of flavor is one of the most important factors in producing a constant-quality beverage. Acetate esters, such as isoamyl acetate (banana-like aroma) and ethyl acetate (solvent-like aroma), have long been recognized as important flavor determinants in beers, being present at or near their sensory thresholds (7). During fermentation, acetate esters are formed by the action of the yeast *Saccharomyces cerevisiae*, but the regulation mechanism of acetate ester synthesis has not yet been completely clarified.

One of the most interesting features of acetate ester synthesis by *S. cerevisiae* is that the production of acetate esters is greatly reduced by aeration or the addition of unsaturated fatty acids to the medium, although the production of higher alcohols, which are the precursor of acetate esters, was not (15, 17, 23, 26). Very low levels of equivalent acetate esters are produced under highly aerobic conditions. Because aeration is a very effective method to accelerate yeast cell growth and fermentation (24), it is very useful to clarify the mechanism of this dramatic reduction in acetate esters for the production of alcoholic beverages.

Alcohol acetyltransferase (AATase, EC 2.3.1.84), which synthesizes a variety of acetate esters from alcohols and acetyl coenzyme A (CoA) (17, 18, 20, 25, 26), has been suggested to be responsible for this phenomenon. Many studies have shown that the AATase activity of *S. cerevisiae* is greatly reduced by aeration or addition of unsaturated fatty acids (8, 17, 26). In addition, it has also been reported that the partially purified AATase is inhibited by unsaturated fatty acids in vitro (20, 25). These results suggest that the unsaturated fatty acid content of the membrane is increased by aeration or unsaturated fatty acid addition, and this causes the inhibition of AATase activity and the reduction in acetate ester synthesis (26).

However, it has not yet been clarified whether the expression level of the AATase gene *ATF1* is affected by aeration or unsaturated fatty acids. We have studied the regulation of the *ATF1* gene by Northern analysis. Our results clearly indicated that expression of the *ATF1* gene is greatly reduced by aeration and by the addition of unsaturated fatty acids. The degree of reduction in AATase activity and in the level of *ATF1* mRNA were closely related, either by aeration or the addition of unsaturated fatty acids. In addition, when the *ATF1* promoter was replaced with a constitutive promoter, no reduction of AATase activity was observed after aeration or the addition of unsaturated fatty acids.

These results suggest that repression of *ATF1* is one of the primary factors producing the reduction in AATase activity. The possible mechanism of *ATF1* repression is also discussed.

MATERIALS AND METHODS

Strains. *S. cerevisiae* TD4 (*MAT***a** *his4-519 ura3-52 leu2-3 leu2-112 trp1 can*), which was confirmed to have one copy of the *ATF1* gene by Southern analysis (data not shown), was used as a wild-type strain.

For the purpose of YEp and YCp plasmid transformation, the *atf1*::*URA3* disruptant of TD4 (9) was used as a host.

S. cerevisiae SH2676 (*MAT***a** *his4-519 ura3-52 leu2-3 leu2-112 trp*), which was a kind gift of S. Harashima, was used for transformation with the *ATF1* promoter-controlled *lacZ* gene.

E. coli DH5 (F$^-$ *deoR supE44 hsdR17 recA1 endA1 gyr96* λ$^-$ *thi-1 relA1*) was used as a host for general plasmid construction.

Culture conditions. Standing cultures and shaking cultures (120 rpm) in 100 ml of YM15 medium (15% dextrose, 1.25% yeast extract, 1.25% malt extract) contained in 500-ml flasks were used to produce the "minimally aerobic" and "highly aerobic" cultures. SD15 medium (15% dextrose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base without amino acids and ammonium sulfate [Difco]) supplemented with amino acids was used as the selective medium.

To investigate *ATF1* gene expression during fermentation, the evolution of both AATase activity and that of *ATF1* mRNA were compared. Yeast cells grown under minimally aerobic or highly aerobic conditions were harvested every 12 h. AATase activity was assayed, and the level of *ATF1* mRNA was estimated by Northern analysis.

A short time course assay to compare the decreases in AATase activity and in the level of *ATF1* mRNA over time was carried out with cells at the start of logarithmic phase (9 h) which had been grown under minimally aerobic conditions and then transferred to highly aerobic conditions or to minimally aerobic conditions with addition of unsaturated fatty acids (1.0 mM sodium salt). The cells were harvested and washed with sterile distilled water and then divided into two aliquots and stored at −80°C until required. One aliquot was used for the AATase assay, and the other was used for Northern analysis.

Plasmid construction. The constitutive *ATF1* expression vector was constructed as follows. The *Nru*I-*Nru*I fragment of YATL1, which has the *ATF1* coding region, was cloned into pBluescriptII KS+ (Stratagene). Using Exonuclease III (Takara), the 5′ upstream region of the *ATF1* gene was deleted. The fragment with the *ATF1* open reading frame, the 26-bp upstream region, and the terminator was cloned into the YCp plasmid pRS415 (Stratagene) under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH [14]) promoter.

The hybrid *ATF1* upstream promoter elements and *CYC1* TATA plasmid were

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constructed as follows. The 5′ upstream region of the *ATF1* gene was amplified by PCR. The amplified fragment inserted into the *Xho*I site of the pLG669-Z, which has the TATA element and the N-terminal encoding region of *CYC1* but not the transcriptional enhancer element (12).

Northern analysis. Total RNA was isolated according to the method of Elion and Warner (6). RNA samples were denatured in glyoxal-dimethyl sulfoxide and subjected to electrophoresis in 1.0% agarose with 10 mM phosphate buffer (pH 7.0), with constant buffer circulation. Northern blotting was performed with GeneScreen Plus (Du Pont) according to the manufacturer's instructions. The 401-bp *Cla*I-*Eco*RI fragment of the *ATF1* gene (8) was used as a probe. The 1,140-bp *Cla*I-*Xho*I fragment of the *ACT1* gene (11) was used as a probe for the internal marker. Labeling of these fragments was carried out with [α-^{32}P]dCTP (NEN), using the Megaprime DNA labeling system (Amersham), according to the manufacturer's instructions. All experiments were carried out in duplicate with essentially identical results (standard margin of error was less than 5%).

Enzyme assays. Yeast cell extracts for the AATase assay were prepared as previously described (8), and AATase activity was determined as described by Minetoki et al. (20). Yeast cell β-galactosidase assays were performed essentially as previously described (2). All enzyme assays were carried out in triplicate, with a standard margin of error less than 3%.

Lipid extraction and analysis. Yeast cells were collected by centrifugation and washed three times with distilled water. Lipid extracts were prepared by adding 30 ml of chloroform-methanol (1:2) to the frozen cell pellet. The mixture was left to stand for 30 min at 4°C, and subsequently 12 ml of distilled water and 12 ml of chloroform were added. The extracts were vortexed, and after centrifugation, the organic phase was collected. The organic phase was dried up by evaporation and then resuspended in 2 ml of chloroform-methanol (2:1). BHT (2,6-di-*t*-butyl-4-methylphenol) was added to the samples and stored at −20°C.

Individual phospholipids were separated by two-dimensional thin-layer-chromatography with chloroform–methanol–28% ammonia (65:35:5, by volume) for development in the first direction and with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by volume) for the second direction. Lipid spots were scraped off, and the lipids were esterified for 150 min at 85°C with 3 ml of 5% HCl in methanol. The resultant methyl esters were analyzed with a gas chromatography system (GC-17A; Shimadzu) equipped with a capillary column QUADREX23 (Quadrex) and a hydrogen flame ionization detector. All experiments were carried out in triplicate, with a standard margin of error less than 3%.

RESULTS

The evolution of AATase activity and *ATF1* mRNA under minimally and highly aerobic conditions. To study the effect of aeration on *ATF1* gene expression during fermentation, we used a standing culture and a shaking culture as models of minimally aerobic and highly aerobic cultures, respectively. The evolution of the AATase activity and that of *ATF1* mRNA under these conditions are compared in Fig. 1.

As the yeast cells were precultured in highly aerobic cultures, the level of AATase activity was initially very low in both cultures. Under minimally aerobic conditions, the degree of AATase activity increased as the cells grew, and the highest level of activity was observed at 12 h of culture, when the cells were in the early logarithmic phase (Fig. 1). At this point, the AATase activity was approximately equal to fourfold the initial activity. After this point, the AATase activity decreased, and when the cells reached stationary phase, the degree of activity had almost returned to the initial level.

Under highly aerobic conditions, AATase activity was repressed over the entire time course, although cell growth was almost identical in the two cultures until 12 h of incubation (Fig. 1B). The activity reached its maximum level, which was 1.6-fold the initial level, after 12 h of culture.

The results of Northern analysis clearly indicated that the level of *ATF1* mRNA increased and decreased in a manner similar to that of AATase activity. Initially, little *ATF1* mRNA was observed. Under minimally aerobic conditions, the maximum level of *ATF1* mRNA was observed after 12 h of cultivation, approximately 33-fold the initial level. Then, it decreased gradually to the original level. In addition, the level of mRNA was greatly reduced under highly aerobic conditions. These data strongly suggest that the expression of *ATF1* mRNA was affected by aeration.

It is reasonable that the repression ratio of the *ATF1* mRNA level is greater than that of the AATase activity; this is because *S. cerevisiae* cells have a minor (10 to 20%) AATase activity which differs from the Atf1 protein and is not repressed by aeration (9).

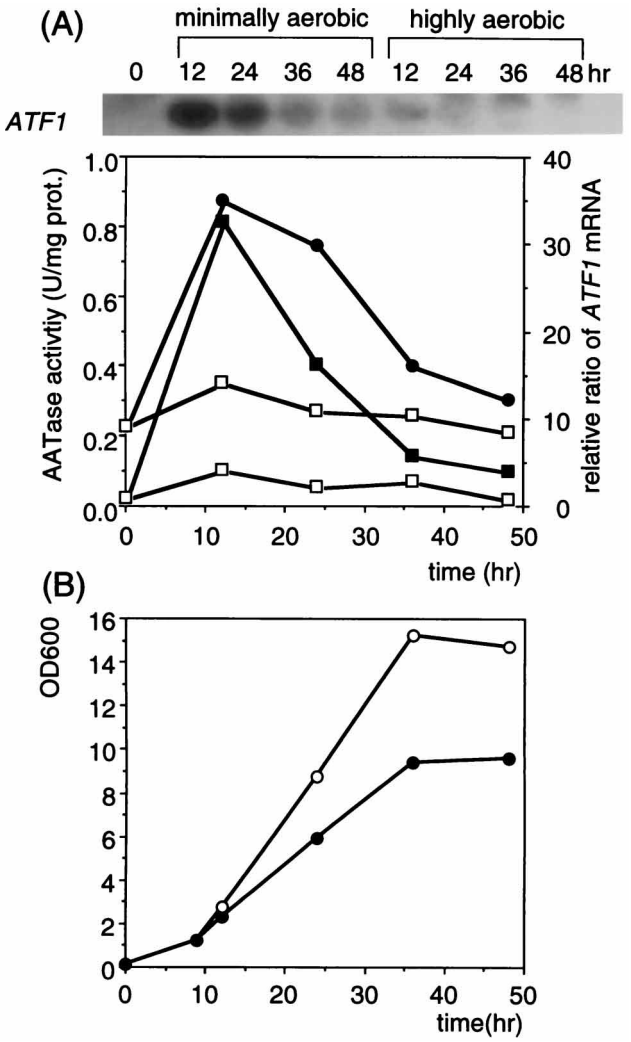

FIG. 1. The evolution of AATase activity and *ATF1* mRNA by *S. cerevisiae* TD4. (A) Open and closed circles represent the degree of AATase activity under highly and minimally aerobic conditions, respectively. Open and closed squares represent the relative amount of *ATF1* mRNA under highly and minimally aerobic conditions, respectively. The results of Northern blotting are shown at the top of the panel. (B) Yeast cell growth under highly (open circles) and minimally (closed circles) aerobic conditions. OD600, optical density at 600 nm.

Short time course assay of AATase activity and *ATF1* mRNA production. To study the relationship between the reduction in the level of *ATF1* mRNA and that of the AATase activity in greater detail, short time course experiments were carried out.

Two yeast cultures were incubated under minimally aerobic conditions until the cells reached the beginning of log phase (9 h, optical density at 600 nm = 1.0). At this growth stage (0 h) (Fig. 2), the AATase activity and the *ATF1* mRNA level began to increase. Then, one of these cultures was transferred to highly aerobic conditions. The other culture was maintained under minimally aerobic conditions and was used as a control. It was clear that under minimally aerobic conditions (control culture), both the level of AATase activity and the *ATF1*

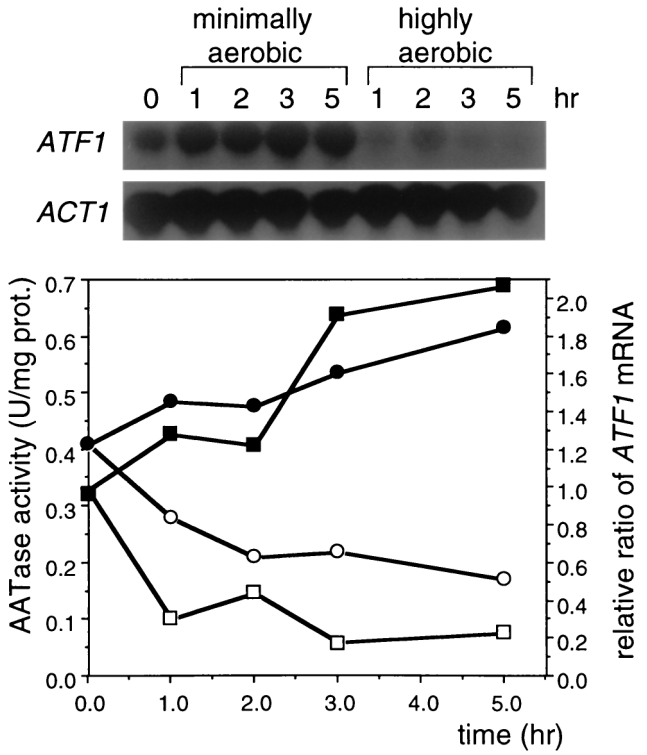

FIG. 2. Reduction in the levels of AATase activity and *ATF1* mRNA by aeration. Yeast cells were cultured for 9 h under minimally aerobic conditions and then transferred to highly or minimally aerobic cultures. The results of Northern blotting are shown at the top of the figure. The amount of mRNA was determined by using *ACT1* mRNA as an internal control. Open and closed circles represent AATase activity under highly and minimally aerobic conditions, respectively. Open and closed squares represent the relative amount of *ATF1* mRNA under highly and minimally aerobic conditions, respectively.

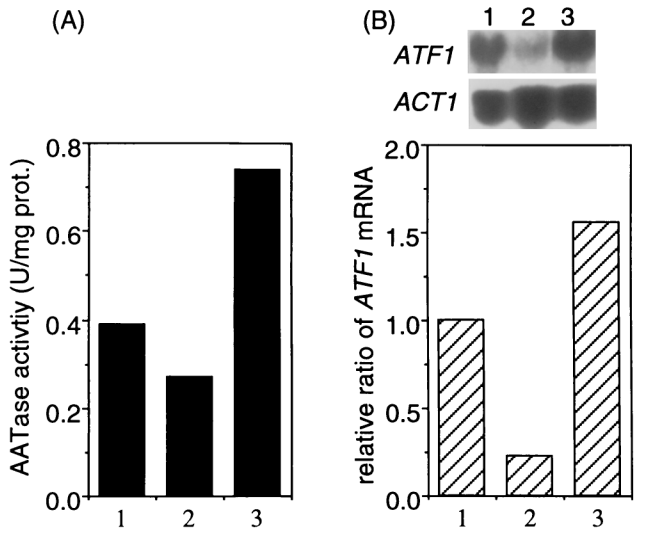

FIG. 3. The effect of air and nitrogen gas on the level of AATase activity (A) and *ATF1* mRNA (B) in shaking culture. Yeast cells were cultured for 9 h under minimally aerobic conditions and then transferred either to a highly aerobic culture or to a shaking culture with nitrogen gas passed through. The two cultures were incubated for 2 h. (A) AATase activity. Samples (bars): 1, before cell transfer; 2, highly aerobic culture; 3, shaking culture with nitrogen gas passed through. (B) The results of Northern blotting are shown at the top of the panel. The amount of mRNA was determined by using *ACT1* mRNA as an internal control. The sample numbers are as described for panel A.

mRNA level continued to increase during the incubation period (Fig. 2).

On the contrary, both AATase activity and the level of *ATF1* mRNA began to decrease within 1 h of incubation under highly aerobic conditions. The low level of activity and that of the mRNA were maintained during the entire incubation time, and the maximum difference in the AATase activity and the *ATF1* mRNA level for the two cultures was observed 5 h after the start of incubation. At that time, AATase activity and the level of *ATF1* mRNA in the highly aerobic culture were reduced to 28 and 12% of the control culture values, respectively. The reductions in AATase activity and *ATF1* mRNA level were shown to have a correlation again; this suggested that the reduction in AATase activity under highly aerobic conditions resulted from repression of the *ATF1* gene.

To confirm that these reductions in AATase activity and in the *ATF1* mRNA level under highly aerobic conditions were caused by aeration and not by the mechanical stress of shaking, nitrogen gas was passed through the shaking culture instead of air. As we expected, AATase activity and the level of *ATF1* mRNA did not decrease but increased after 2 h of shaking (Fig. 3). This suggests that oxygen is the primary factor repressing *ATF1* expression.

It has been reported that the unsaturated fatty acid content of yeast cells grown under anaerobic conditions is less than that of yeast cells grown under highly aerobic conditions (15). To study the fatty acid composition of yeast cells under our conditions, lipid analysis was carried out. Glycerolipids were extracted from the cells cultured for 9 h under minimally aerobic conditions (0 h), 1 h after incubation under highly aerobic conditions, and 1 h after incubation under minimally aerobic conditions. The fatty acid compositions of total lipid, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) molecules were determined.

The results suggested that even under minimally aerobic conditions, unsaturated fatty acids (14:1, 16:1, and 18:1) accounted for almost 72% of total lipids, 80% of PC, and 60% of PE (Table 1). In addition, little change in fatty acid composition was observed after 1 h of aeration, even though repression of *ATF1* mRNA was observed (Fig. 2). This suggests that the unsaturated fatty acid composition of the yeast cell membrane

TABLE 1. Fatty acid composition of yeast cells[a]

Lipid	Condition	Time (h)	Fatty acid composition (%)						
			14:0	14:1	16:0	16:1	18:0	18:1	18:2
Total		0	3.1	0.6	20.7	41.5	3.8	30.3	0.0
	Minimally aerobic	1	2.9	0.6	22.4	40.2	4.3	29.4	0.0
	Highly aerobic	1	2.7	0.6	19.0	41.8	4.0	31.9	0.0
	Linoleic acid	1	2.2	0.2	16.3	16.0	3.0	11.1	51.2
PC		0	2.8	0.7	15.2	55.9	2.1	23.3	0.0
	Minimally aerobic	1	2.5	0.6	16.9	55.7	2.7	21.6	0.0
	Highly aerobic	1	1.9	0.6	13.9	57.5	2.6	23.5	0.0
	Linoleic acid	1	1.8	0.3	16.5	26.6	3.2	10.1	41.5
PE		0	1.8	0.0	27.0	36.3	1.2	33.7	0.0
	Minimally aerobic	1	1.3	0.0	26.4	34.1	1.6	36.6	0.0
	Highly aerobic	1	1.7	0.0	24.4	36.7	1.4	35.8	0.0
	Linoleic acid	1	1.4	0.0	31.9	20.8	1.2	15.7	29.0

[a] Yeast cells were cultured for 9 h under minimally aerobic conditions (0 h) then transferred to cultures with the indicated conditions for 1 h.

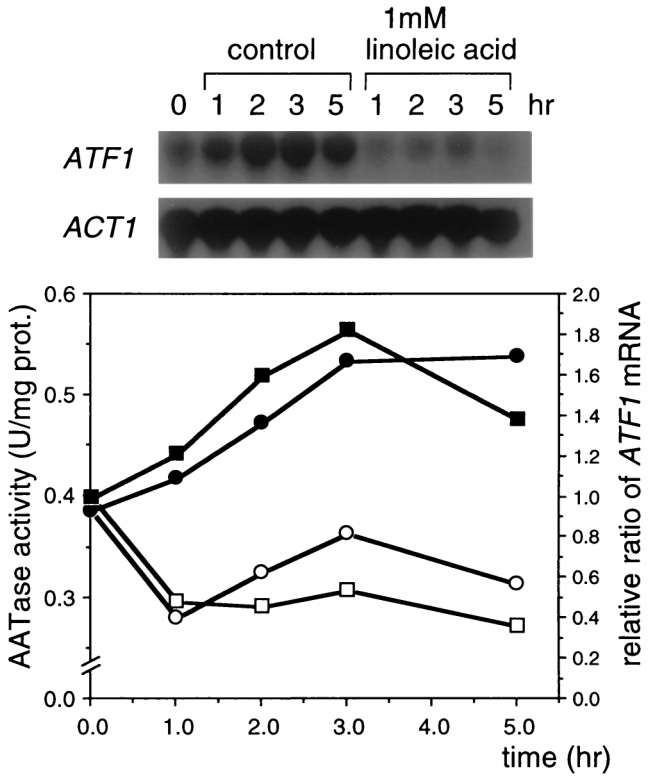

FIG. 4. Reduction in the levels of AATase activity and *ATF1* mRNA by unsaturated fatty acid addition. Yeast cells were cultured for 9 h under minimally aerobic conditions and then transferred to minimally aerobic cultures with or without 1 mM sodium linoleic acid. The results of Northern blotting are shown at the top of the figure. The amount of mRNA was determined by using *ACT1* mRNA as an internal control. Open and closed circles represent the level of AATase activity in the presence and in the absence of 1 mM sodium linoleic acid, respectively. Open and closed squares represent the relative amount of *ATF1* mRNA activity with and without 1 mM sodium linoleic acid, respectively.

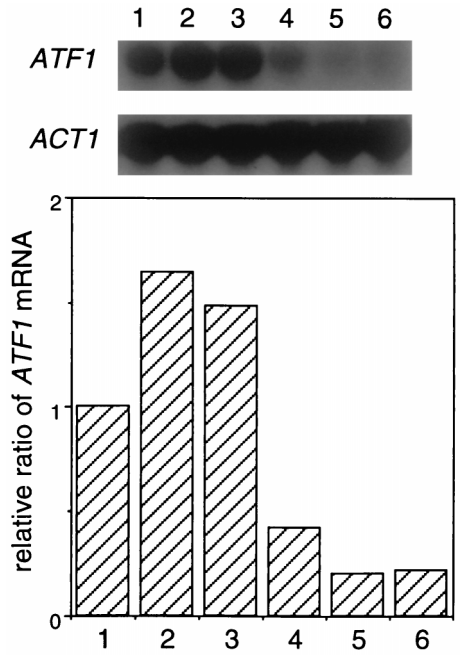

FIG. 5. Reduction in the level of *ATF1* mRNA by various fatty acid addition. Yeast cells were cultured for 9 h under minimally aerobic conditions and then transferred to minimally aerobic cultures with 1 mM fatty acid and incubated for 3 h. The results of Northern blotting are shown at the top of the figure. The amount of mRNA was determined by using *ACT1* mRNA as an internal control. Samples (bars): 1, before cell transfer; 2, minimally aerobic conditions; 3, minimally aerobic conditions with 1 mM stearic acid; 4, minimally aerobic conditions with 1 mM oleic acid; 5, minimally aerobic conditions with 1 mM linoleic acid; 6, minimally aerobic conditions with 1 mM linolenic acid.

is not a factor which triggers the reduction of *ATF1* mRNA by aeration.

Effect of unsaturated fatty acids on *ATF1* gene expression. Like aeration, the addition of unsaturated fatty acids to the medium has been reported to cause a reduction in AATase activity (17, 26). To confirm that the *ATF1* gene was repressed by unsaturated fatty acids in addition to aeration, a short time course assay of AATase activity and *ATF1* mRNA analysis was carried out. Two minimally aerobic cultures were incubated for 9 h, and then linoleic acid (18:2) was added to one of these cultures to a final concentration of 1 mM. Both cultures were maintained under minimally aerobic conditions during the entire incubation.

Reduction in both the AATase activity and the *ATF1* mRNA level were observed within 1 h of linoleic acid addition, and these levels were maintained during the incubation (Fig. 4). The maximum difference for the two cultures was observed 5 h after the addition of unsaturated fatty acids (approximately 58% of that of the control). The maximum difference in the level of *ATF1* mRNA was observed 3 h after unsaturated fatty acid addition. The level of mRNA produced by the *ATF1* gene in the presence of unsaturated fatty acids was about 26% of that of the control cells after 5 h of incubation. The reduction in AATase activity and in the level of *ATF1* mRNA did not result from cell growth inhibition, because yeast cell growth was not affected by the presence of unsaturated fatty acids in this experiment (data not shown).

Lipid analysis suggested that under these conditions, linoleic acid was efficiently incorporated into the yeast cell membrane. After 1 h, the linoleic acid accounted for 51% of the total lipids, 42% of PC, and 29% of PE (Table 1). The level of other unsaturated fatty acids (16:1 and 18:1) was drastically reduced (45% in total lipids); this was caused by the repression of the Δ-9 desaturase gene *OLE1*, as previously reported (3, 19), but the decrease of saturated fatty acids was small (5% in total lipids).

To determine which fatty acids repress *ATF1* expression, several types of fatty acids were added to minimally aerobic cultures, and the levels of *ATF1* mRNA were determined.

A similar repression of *ATF1* mRNA was observed with oleic acid (18:1) and linolenic acid (18:3) but was not observed with stearic acid (18:0). The *ATF1* mRNA levels in cells grown with oleic acid, linoleic acid, and linolenic acid were 26, 12, and 13% of that of the control cells, respectively (Fig. 5). This suggests that only unsaturated fatty acids repress *ATF1* gene expression and that saturated fatty acids have no effect.

Expression of the *ATF1* gene with a constitutive promoter. The results presented above strongly suggest that the repression of the *ATF1* gene is one of the primary factors in the reduction of AATase activity. In order to clarify whether AATase is regulated by posttranscriptional modification in vivo, we constructed a YCp plasmid which expressed the *ATF1* gene constitutively from the GAPDH promoter. The *atf1*::*URA3* disruptant was transformed with this plasmid, and the levels of AATase activity of the transformants were measured under minimally aerobic and highly aerobic condi-

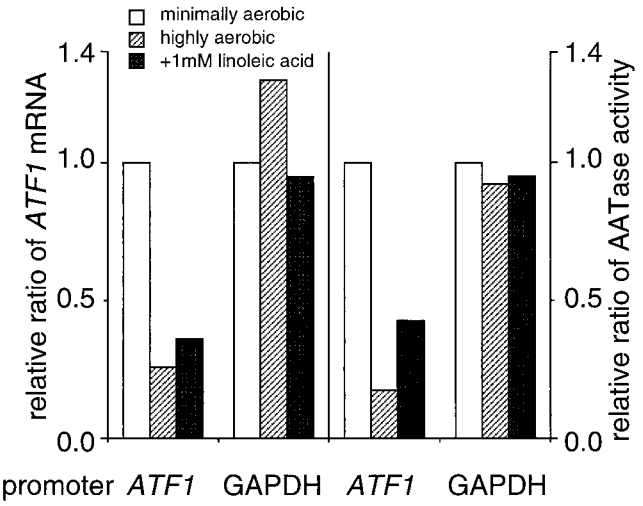

FIG. 6. Comparison of the levels of *ATF1* mRNA and AATase activity expressed from the *ATF1* and GAPDH promoters. Yeast cells were cultured for 9 h under minimally aerobic conditions and then transferred to cultures with the indicated conditions and incubated for 3 h. The amount of mRNA was determined by using *ACT1* mRNA as an internal control.

tions and under conditions in which unsaturated fatty acids were present. As a control, the *atf1*::*URA3* disruptant with the YEp plasmid which expresses the *ATF1* gene from the native promoter was used. Each culture was incubated for 9 h under minimally aerobic conditions and then switched to the appropriate conditions and incubated for 3 h.

Northern analysis clearly showed that the level of *ATF1* mRNA was not reduced either by aeration or by the addition of unsaturated fatty acids when the *ATF1* gene was driven by the GAPDH promoter. In addition, the level of AATase activity was kept almost the same under highly aerobic conditions and under conditions in which unsaturated fatty acids were present (Fig. 6). In the case of the native promoter, the AATase activities of the highly aerobic culture and of the culture in which unsaturated fatty acids were present were 18 and 43% of that of the control, respectively. These results suggest that repression of the *ATF1* gene is the primary factor in the reduction of AATase activity by aeration and unsaturated fatty acid addition.

***lacZ* expression driven by the *ATF1* promoter.** In our previous work, we found that *Saccharomyces carlsbergensis* has another type of *ATF1* gene (Lg-*ATF1*) which was repressed as well as the *ATF1* gene by aeration and by unsaturated fatty acids (10). The sequence comparison of the *ATF1* and the Lg-*ATF1* gene suggested that only a limited region of the 5′ flanking region (−196 to −60) seems to be homologous (77%).

To determine whether this homologous 5′ flanking region was responsible for repression of the *ATF1* gene, an experiment using the *E. coli lacZ* gene as a reporter was carried out. The plasmid pLG669-Z was used for this purpose. This plasmid has the TATA element and the N-terminal encoding region of *CYC1* fused to the *E. coli lacZ* gene. The upstream region (−200 to −50) of the *ATF1* gene was amplified by PCR and inserted into the *Xho*I site of pLG669-Z.

The β-galactosidase activity of the *ATF1-CYC1* hybrid plasmid shows much greater activity under minimally aerobic conditions than the vector without the *ATF1* 5′ sequence. The activity was strongly repressed under both highly aerobic conditions and conditions in which unsaturated fatty acids were present (Table 2). This result suggests that the repression of

TABLE 2. β-Galactosidase activity driven by 5′ flanking sequence of *ATF1*

Plasmid	Activity (U) under condition:		
	Minimally aerobic	Highly aerobic	+1 mM linoleic acid
ATF1	197 (1.0)	18 (0.09)	9 (0.05)
pLG669-8	<1	<1	<1

the *ATF1* gene by aeration and unsaturated fatty acids occurred by transcriptional control and that the 150-bp upstream sequence had an important role in these repression systems.

DISCUSSION

The reduction of AATase activity by aeration or by unsaturated fatty acids has been of interest for many years (17, 20, 26). Because it has been well known that oxygen is necessary for the synthesis of unsaturated fatty acids in *S. cerevisiae* (1), aeration and unsaturated fatty acids have been speculated to reduce the AATase activity by the same mechanism. Previous reports have also suggested that AATase was inhibited by high concentrations (2 mM) of unsaturated fatty acids in vitro (20, 25). However, because unsaturated fatty acids act as a surfactant and are able to inactivate many enzymes in vitro (21), it is possible that the reduction mechanism in vivo is different. Malcorps and Dufour (17) have suggested that gene repression might be related to the reduction of AATase activity, but molecular analysis was not carried out.

In this report, we have described the regulation of the *ATF1* gene by aeration and unsaturated fatty acids. The Northern analysis data clearly indicated that expression of the *ATF1* gene is regulated by aeration. The increase and decrease in the level of AATase activity under minimally aerobic conditions were very similar to the changes observed for *ATF1* mRNA.

The results of the short time course experiments suggested that the reduction in AATase activity produced by aeration occurred within 1 h of cultivation. Again, the repression in the level of *ATF1* mRNA was closely related to that of the level of AATase activity. Similar results were obtained when unsaturated fatty acids were added to the culture instead of aeration. No reduction in AATase activity or in the level of *ATF1* mRNA was observed when nitrogen gas was passed through the culture instead of air.

When the promoter of the *ATF1* gene was replaced with the constitutive GAPDH promoter, both AATase activity and the *ATF1* mRNA level were unaffected by aeration or unsaturated fatty acids. The experiments using the *lacZ* reporter gene suggested that repression by aeration and unsaturated fatty acids was controlled at the transcriptional level. The results of this experiment suggested that the 150-bp 5′ flanking sequence (−50 to −200) might have an important role in both types of regulation. These results suggest that the repression of the *ATF1* gene is one of the most important factors causing the reduction of AATase activity in vivo.

Although the biological function of the *ATF1* gene has not been clarified, our results make it easy to speculate that it has some relationship with unsaturated fatty acid metabolism. Interestingly, the *OLE1* gene of *S. cerevisiae*, which encodes a Δ-9 acyl-CoA desaturase and has a major role in unsaturated fatty acid synthesis in yeast cells (22), has been reported to be transcriptionally repressed by unsaturated fatty acids (3, 19). The *OLE1* gene is repressed not only by unsaturated fatty acids

which occur naturally in the natural yeast membranes (16:1 and 18:1) but also by unsaturated fatty acids which do not occur naturally in the yeast membrane (18:2 and 18:3). Our data indicated that the *ATF1* gene was also repressed by the 18:1, 18:2, and 18:3 unsaturated fatty acids to a similar degree. It is possible that a common transcriptional repressor is responsible for unsaturated fatty acid repression of both the *ATF1* gene and the *OLE1* gene.

Gene regulation by fatty acids has been reported not only for yeast but also for bacteria (13) and mammalian cells (5, 16). In *E. coli*, fatty acid has to be converted to an acyl-CoA derivative in order to repress the *fabA* gene (13). An important issued to be resolved is which molecule acts as a sensor in *ATF1* gene regulation by unsaturated fatty acids.

Our Northern analysis results suggest that repression of the *ATF1* gene by unsaturated fatty acids is similar to that caused by aeration. Both types of repression occurred within 1 h of treatment. However, it is not clear whether the *ATF1* gene has a sensor common to both factors. Lipid analysis data showed that only minor changes were observed in the fatty acid composition of the yeast cell 1 h after aeration, suggesting that change in fatty acid composition is not a necessary factor in *ATF1* gene repression by aeration.

Many genes are known to be regulated by oxygen using heme as a regulation signal (27). Interestingly, a recent study suggested that the expression level, but not unsaturated fatty acid repression, of the *OLE1* gene was affected by the presence of the *HAP1* gene, which is a regulator of the heme-related genes (4). Aeration may therefore possibly repress the *ATF1* gene by using a sensor, such as heme, which is different from that used by unsaturated fatty acids.

In conclusion, our work suggests that *ATF1* is a good model to study the regulation of the yeast system by aeration and unsaturated fatty acids. Further study of *ATF1* gene regulation will also provide us with a new means of counteracting the effects of aeration and unsaturated fatty acid addition on acetate ester production during fermentation by the application of genetic engineering or classical mutation methods.

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