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Molecular Cloning, Sequence Analysis, and Expression of the Yeast Alcohol Acetyltransferase Gene

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The *ATF1* gene, which encodes alcohol acetyltransferase (AATase), was cloned from *Saccharomyces cerevisiae* and brewery lager yeast (*Saccharomyces uvarum*). The nucleotide sequence of the *ATF1* gene isolated from *S. cerevisiae* was determined. The structural gene consists of a 1,575-bp open reading frame that encodes 525 amino acids with a calculated molecular weight of 61,059. Although the yeast AATase is considered a membrane-bound enzyme, the results of a hydrophobicity analysis suggested that this gene product does not have a membrane-spanning region that is significantly hydrophobic. A Southern analysis of the yeast genomes in which the *ATF1* gene was used as a probe revealed that *S. cerevisiae* has one *ATF1* gene, while brewery lager yeast has one *ATF1* gene and another, homologous gene (Lg-*ATF1*). Transformants carrying multiple copies of the *ATF1* gene or the Lg-*ATF1* gene exhibited high AATase activity in static cultures and produced greater concentrations of acetate esters than the control.

Acetate esters, such as isoamyl acetate and ethyl acetate, are recognized as important flavor compounds in beer and other alcoholic beverages. It has been suggested that alcohol acetyltransferase (AATase) (EC 2.3.1.84) is one of the most important enzymes for acetate ester formation. AATase is an SH enzyme which reacts with acetyl coenzyme A and, depending on the degree of affinity, with various kinds of alcohols (22). The activity of this enzyme is strongly repressed under aerobic conditions or by the addition of unsaturated fatty acids to a culture (10, 13, 22).

As acetate esters affect the flavor quality of alcoholic beverages, many workers have attempted to clone the AATase gene in order to understand the mechanism of acetate ester synthesis and to control ester production (11, 13, 22).

Recently, we succeeded in purifying this enzyme from *Saccharomyces cerevisiae* to homogeneity and determined its internal peptide sequences (13). The molecular weight of this enzyme was estimated to be about 60,000. In this paper, we describe the cloning of the AATase-encoding gene. We cloned the gene from two yeast phage libraries, one constructed with *S. cerevisiae* DNA and the other constructed with DNA from brewery lager yeast (*Saccharomyces uvarum*). The nucleotide sequence of the *ATF1* gene isolated from *S. cerevisiae* revealed that the molecular weight of the encoded protein is 61,059. A Southern analysis of yeast genomes revealed that *S. cerevisiae* has one *ATF1* gene, but brewery lager yeast has one *ATF1* gene and another homologous gene. We also obtained expression of these genes by using a multicopy plasmid. The resulting transformants exhibited 6- to 15-fold-greater AATase activity than the control. The concentrations of acetate esters present in cultured supernatants obtained from transformant cultures were also greater than the concentrations present in the control.

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MATERIALS AND METHODS

Strains and plasmid. *S. cerevisiae* Kyokai No. 7 (=K7 = IFO 2347) was obtained from the Institute for Fermentation, Osaka, Japan. Brewery lager yeast strain KBY001 (*S. uvarum*) was obtained from our culture collection. *S. cerevisiae* TD4 (*a his4-519 ura3-52 leu2-3 leu2-112 trp can*) was used for transformation and for expression of the *ATF1* multicopy plasmid. *Escherichia coli* DH5 (*endAI gyrA96 hsdR17 recA1 relA1 supE44 thi-1*) was used as a host for plasmid construction. Plasmid YEp13K, which is described elsewhere (19), was used as a vector for yeast plasmid construction.

Media. For growth under selective conditions we used SD medium (2% dextrose, 0.67% yeast nitrogen base [Difco]) containing amino acid supplements. YM15 medium (15% dextrose, 1.25% yeast extract [Difco], 1.25% malt extract [Difco]) was used for cultures grown for AATase assays and for determinations of ester concentrations.

Genomic DNA isolation. Yeast genomic DNA for library construction was isolated as described by Rose and Broach (16). Small-scale preparations of yeast DNA were obtained by the method of Rose et al. (17).

Construction and screening of the libraries. Yeast genomic DNA was partially digested with *Sau*3AI to give fragments with an average size of 15 to 20 kb. These fragments were ligated into the *Bam*HI site of the λ-EMBL3 vector (Stratagene) and packaged in vitro (Stratagene). *E. coli* P2392 {F^- *galK2 galT22 hsdR514* [r_k^- m_k^+] λ^- *lacYI* or Δ(*lacIZY*)6 *mcrA mcrB*$^+$ *metB1 P2 supE44 supF58 trpR55*} was infected with the recombinant phage.

Two peptides were chosen for construction of two oligonucleotide mixed probes. The oligonucleotide mixed probes were synthesized with an Applied Biosystems model 380B instrument. The oligonucleotides were purified with oligonucleotide purification cartridges (Applied Biosystems) and were labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. For the initial screening, 30,000 recombinant phages from the λ-EMBL3 library were plated onto *E. coli* P2392. Duplicate transfers of the clones were made onto nylon membranes. The filters were prehybridized for 3 h at 60°C and hybridized for 18 h at 30°C

in a solution containing 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 10 μg of salmon sperm DNA (Sigma) per ml. Approximately 500,000 cpm of 5′-end-labelled oligonucleotide mixture per filter was used. The filters were washed twice with a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min at 30°C.

To clone the *ATF1* gene from brewery lager yeast strain KBY001, a 0.4-kb *Cla*I-*Eco*RI fragment of the *S. cerevisiae ATF1* gene was used as a probe after labelling with [α-^{32}P]dCTP using a multiprime labelling kit. The filters were hybridized at 65°C for 18 h, washed twice with a solution containing 2× SSC and 0.1% SDS, and then washed twice with 0.5× SSC for 30 min at 65°C.

Transformation. *E. coli* transformation was carried out as described by Hanahan (5), using frozen competent cells obtained from Toyobo, Osaka, Japan. Transformation of *S. cerevisiae* strains was carried out by the lithium acetate procedure (8).

DNA sequencing. *E. coli* JM109 {Δ(*lac-proAB*) *endA1 gyrA96 hsdR17 thi* λ$^-$ *relA1 supE44* [F′ *traD36 proAB lacI^{q}ZΔM15*]} and bacteriophage M13mp18,mp19 (21) were used to clone DNA fragments for sequencing. Sequencing was performed by the dideoxy chain termination method (18), using a *Bst* DNA polymerase sequencing kit obtained from Bio-Rad Laboratories according to the supplier's instructions. Chemically synthesized sequencing primers were used when it was not practical to use the M13 universal primer. The sequences obtained were analyzed with the DNASIS program (Hitachi Software Engineering Co., Ltd., Yokohama, Japan).

AATase assay. To measure the AATase activity of yeast cells, cultures were grown in 100 ml of YM15 medium in 500-ml flasks at 30°C. The cultures were grown either with shaking (120 rpm) or under static conditions. To prepare a yeast cell extract, all procedures were performed at 4°C or on ice. Yeast cells were harvested and washed once with distilled water and once with buffer A (25 mM imidazole-HCl, 0.1 M NaCl, 20% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, 0.5% isoamyl alcohol; pH 7.5). The yeast pellet was resuspended in 0.25 ml of buffer A. Then 1.5 g of glass beads was added, and the mixture was vortexed. The beads were washed three times with 0.25 ml of buffer A, and then the cell suspensions were centrifuged at 15,000 × *g* for 20 min to remove any unbroken cells. AATase activity was measured as previously described (12). The protein concentration in the homogenate was determined with a protein assay kit (Bio-Rad Laboratories).

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in the GSDB, DDBJ, EMBL, and NCBI databases under accession number D26554.

RESULTS

Cloning of the *S. cerevisiae ATF1* gene. The method used to clone the AATase-encoding gene utilized oligonucleotide probes constructed on the basis of the amino acid sequences obtained for purified *S. cerevisiae* Kyokai No. 7 AATase. The peptide sequences that were determined are shown in Fig. 1. Two synthetic oligonucleotides, probes 2 and 5 (Fig. 1), were prepared and used for the initial screening.

We screened a total of 30,000 recombinants from the λ-EMBL3 library, and we obtained 14 positive clones. The DNA inserts were analyzed by restriction enzyme digestion

peptide 1	(K)WK
peptide 2a	(K)YVNIDK
peptide 2b	(K)VMCDRAIGK
peptide 3	(K)NQAPVQQECL
peptide 4	(K)GMNIVVASWK
peptide 5	(K)YEEDYQLLRK
peptide 6	(K)QILEEFK
peptide 7	(K)LDYIFK
peptide 9	(K)LSGVVLNEQPEY
peptide 10	(K)NVVGSQESLEELCSIYK
peptide 11	(K)RRGGRLLSNVGLFNQLEEP

```
probe 5            K  Y  E  E  D  Y
(peptide 5)   5'-AAATATGAAGAAGATTATCA-3'
                    G  C  G  G  C  C

probe 2            K  Y  V  N  I  D
(peptide 2a)  5'-AAATATGTAAATATTGA-3'
                    G  C  G  C  C
                          C     A
                          T
```

FIG. 1. Peptide sequences and DNA sequences of the synthesized oligonucleotide probes.

and Southern blotting. The results showed that all of the positive clones carried the same gene locus. Figure 2a shows a restriction map of the AATase-encoding gene of *S. cerevisiae* Kyokai No. 7, which was designated *ATF1*. The two synthetic oligonucleotide probes were hybridized to a 1.0-kb *Eco*RI-*Bam*HI fragment in Fig. 2a.

Nucleotide sequence of the *ATF1* gene. The 6.6-kb *Xba*I fragment shown in Fig. 2a was partially sequenced. The resulting sequence is shown in Fig. 3. A computer analysis revealed that the largest open reading frame of the sequenced fragment extended from nucleotide 234 to nucleotide 1808. This open reading frame encoded a 525-amino-acid protein with a molecular weight of 61,059. All 10 peptide sequences which were determined by sequencing the purified AATase (Fig. 1) were present in this predicted protein (Fig. 3). In addition, the molecular weight of the protein was consistent with the value (60,000) estimated by SDS-polyacrylamide gel electrophoresis of the purified enzyme.

From the codon usage data for the *ATF1* gene, a codon bias index of 0.07 was calculated by the method of Bennetzen and Hall (2). This low codon bias index value for *ATF1* suggested that the level of expression of the *ATF1* gene might be very low.

The Atf1 protein contains 14 cysteine residues out of a total of 525 amino acids. This number of cysteine residues is greater than the numbers of cysteine residues in common acetyltransferase and acyltransferase. It is well known that AATase is highly labile, and it is possible that the high number of cysteine residues is partially responsible for the lability of this enzyme.

Figure 4 shows the hydrophobicity profile of the Atf1 protein. The AATase has been recognized as a membrane-bound enzyme, and solubilization with Triton X-100 was necessary to purify the Atf1 protein. However, interestingly, the results of our hydrophobicity analysis of the Atf1 protein indicated that this protein does not have a significantly hydrophobic domain (>30 amino acids); its mean index was calculated to be −0.38 by the method of Kyte and Doolittle (9).

Cloning of the *ATF1* and Lg-*ATF1* genes from brewery lager yeast. Figure 2d shows the results of a Southern blot analysis of yeast genomic DNA digested with either *Cla*I or *Xba*I when the 0.4-kb *Cla*I-*Eco*RI fragment shown in Fig. 2a was used as the probe. In *S. cerevisiae* Kyokai No. 7 only one band was detected under strict hybridization conditions (Fig. 2d, lanes 1 and 3). However, in brewery lager yeast strain KBY001, two bands were detected under the same hybridization conditions (Fig. 2d, lanes 2 and 4). One band was similar in size to the band obtained for *S. cerevisiae*, and it exhibited strong hybridization with the probe. The other band was a different size and exhibited weak hybridization with the probe. These results suggested that *S. cerevisiae* has a unique *ATF1* gene and that brewery lager yeast has one *ATF1* gene and another, homologous gene.

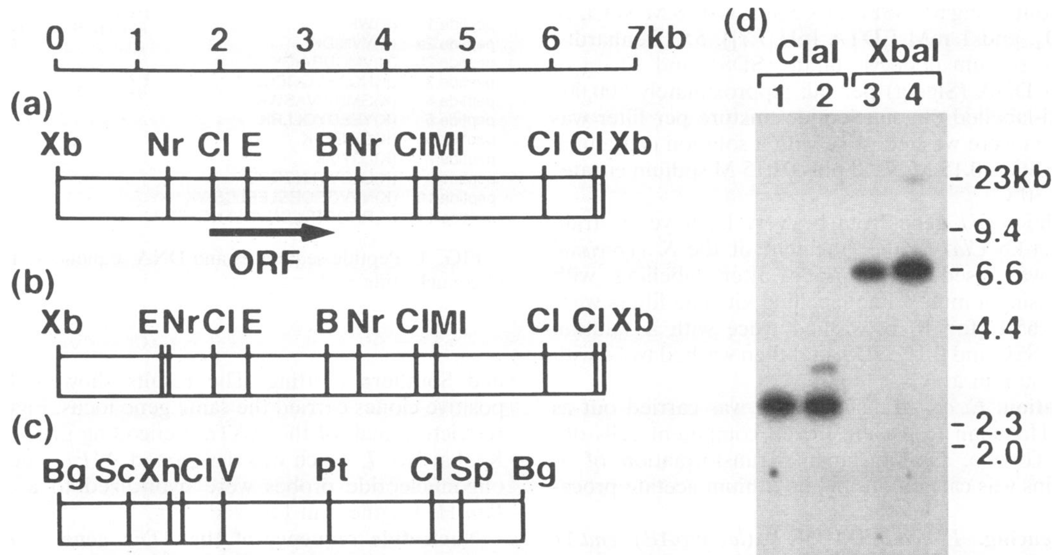

FIG. 2. Restriction maps of the *ATF1* genes. The enzymes used were *Xba*I (Xb), *Eco*RI (E), *Bgl*II (Bg), *Bam*HI (B), *Cla*I (Cl), *Mlu*I (Ml), *Nru*I (Nr), *Hpa*I (Hp), *Pst*I (Pt), *Eco*RV (V), *Xho*I (Xh), and *Sca*I (Sc). (a) Restriction map of the *ATF1* gene originating from *S. cerevisiae* Kyokai No. 7. The position and direction of the 1,575-bp open reading frame (ORF) encoding the AATase are indicated by the arrow. (b) Restriction map of the *ATF1* gene originating from brewery lager yeast (*S. uvarum*). (c) Restriction map of the Lg-*ATF1* gene originating from brewery lager yeast. (d) Southern blot analysis of genomic DNAs from *S. cerevisiae* Kyokai No. 7 (lanes 1 and 3) and brewery lager yeast strain KBY001 (lanes 2 and 4). A 0.4-kb *Cla*I-*Eco*RI fragment of the *ATF1* gene from *S. cerevisiae* Kyokai No. 7 was used as the probe.

We then cloned the AATase-encoding genes from the λ-EMBL3 library constructed from KBY001 DNA by the 0.4-kb *Cla*I-*Eco*RI fragment from the *S. cerevisiae* Kyokai No. 7 *ATF1* gene as the probe. We screened a total of 30,000 recombinants from the λ-EMBL3 library, and we obtained 17 clones which exhibited strong hybridization with the probe and 11 clones which exhibited weak hybridization with the probe.

Figures 2b and c show restriction maps of the DNA inserts which were cloned in the strongly hybridized clones and the weakly hybridized clones, respectively.

It is clear that the strongly hybridized DNA fragment encodes the *ATF1* gene of brewery lager yeast, because its structure is quite similar to the structure of the *ATF1* gene of *S. cerevisiae*.

The weakly hybridized DNA fragment has a different structure. However, because it has been suggested that brewery lager yeast is an allopolyploid and has two sets of genes, which are structurally different but have similar functions (4, 6, 7, 14), we speculated that this homologous gene might be a derivative of the *ATF1* gene. This homologous gene appears to be specific to brewery lager yeast, and we designated this gene the Lg-*ATF1* gene.

Expression of the *ATF1* and Lg-*ATF1* genes in *S. cerevisiae*. To confirm that the three cloned fragments really encode AATase, the 6.6-kb *Xba*I fragment from *S. cerevisiae* Kyokai No. 7 and the 6.6-kb *Xba*I fragment and 5.7-kb *Bgl*II fragment from brewery lager yeast strain KBY001 were subcloned into yeast shuttle vector YEp13K; the resulting plasmids were designated YATK11, YATL1, and YATL2, respectively. These plasmids were used to transform *S. cerevisiae* TD4. Each transformant was grown in a static culture in YM15 medium at 30°C for 24 h, and then AATase activity was measured.

All of the transformants exhibited very high levels of AATase activity, but the levels of activity differed depending on the origin of the gene (Table 1). The *ATF1* gene from brewery lager yeast exhibited the highest level of activity (15 times greater than the control level), and the Lg-*ATF1* gene exhibited the lowest level of activity (6.5 times greater than the control level).

When these transformants were cultured with vigorous shaking, they exhibited very low levels of AATase activity (Table 1). These results indicated that all of the cloned fragments encoded an AATase gene.

Effect of the *ATF1* gene on acetate ester production in yeast cells. AATase is recognized as an enzyme that plays a primary role in acetate ester synthesis in many alcoholic beverages. To evaluate the effect of the cloned *ATF1* and Lg-*ATF1* genes on ester synthesis during fermentation, the transformants and the parental strain were cultured at 30°C for 24 h in YM15 medium, and then the volatile ester concentrations in the culture supernatants were determined.

Compared with the parental strain, the YATL1 transformants exhibited a 27-fold increase in isoamyl acetate production and a 9-fold increase in ethyl acetate production, and the YATL2 transformants exhibited a 17-fold increase in isoamyl acetate production and a 2-fold increase in ethyl acetate production (Table 2).

The production of ethanol and other higher alcohols did not change. These results indicate that AATase activity is a limiting factor in the production of acetate esters in fermented media.

DISCUSSION

Molecular cloning and nucleotide sequence of the *ATF1* gene. The volatile ester concentration is one of the most important characteristics of alcoholic beverages. The ester concentration depends on oxygen (20), CO_2 pressure (15), and other factors (23).

AATase is known to be responsible for acetate ester synthe-

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AGCGTGTGAGGACTACTCATTGGCTTGCGATTTACGGTTTTTATATTTTTTGCCGCACATCATTTTTTGGCCTGGTATTGTCATCGCGTTG   91

AGCGGACTCTGAATATAATCCTATTGTTTTTTATGGATCTCTGGAAGCGTCTTTTTGAAGCCAACCCAACAAAAATTCGAGACAAGAAAAT  182

AAAAAACGGCACTTCATCAGTATCACAAATACCATCAATTTATCAGCTCTC ATG AAT GAA ATC GAT GAG AAA AAT CAG GCC  263
                                                     M   N   E   I   D   E   K   N   Q   A    10

CCC GTG CAA CAA GAA TGC CTG AAA GAG ATG ATT CAG AAT GGG CAT GCT CGG CGT ATG GGA TCT GTT GAA  332
 P   V   Q   Q   E   C   L   K   E   M   I   Q   N   G   H   A   R   R   M   G   S   V   E    33

GAT CTG TAT GTT GCT CTC AAC AGA CAA AAC TTA TAT CGG AAC TTC TGC ACA TAT GGA GAA TTG AGT GAT  401
 D   L   Y   V   A   L   N   R   Q   N   L   Y   R   N   F   C   T   Y   G   E   L   S   D    56

TAC TGT ACT AGG GAT CAG CTC ACA TTA GCT TTG AGG GAA ATC TGC CTG AAA AAT CCA ACT CTT TTA CAT  470
 Y   C   T   R   D   Q   L   T   L   A   L   R   E   I   C   L   K   N   P   T   L   L   H    79

ATT GTT CTA CCA ATA AGA TGG CCA AAT CAT GAA AAT TAT TAT CGC AGT TCC GAA TAC TAT TCA CGG CCA  539
 I   V   L   P   I   R   W   P   N   H   E   N   Y   Y   R   S   S   E   Y   Y   S   R   P   102

CAT CCA GTG CAT GAT TAT ATT TCA GTA TTA CAG GAA TTG AAA CTG AGT GGT GTG GTT CTC AAT GAA CAA  608
 H   P   V   H   D   Y   I   S   V   L   Q   E   L   K   L   S   G   V   V   L   N   E   Q   125

CCT GAG TAC AGT GCA GTA ATG AAG CAA ATA TTA GAA GAA TTC AAA AAT AGT AAG GGT TCC TAT ACT GCA  677
 P   E   Y   S   A   V   M   K   Q   I   L   E   E   F   K   N   S   K   G   S   Y   T   A   148

AAA ATT TTT AAA CTT ACT ACC ACT TTG ACT ATT CCT TAC TTT GGA CCA ACA GGA CCG AGT TGG CGG CTA  746
 K   I   F   K   L   T   T   T   L   T   I   P   Y   F   G   P   T   G   P   S   W   R   L   171

ATT TGT CTT CCA GAA GAG CAC ACA GAA AAG TGG AAA AAA TTT ATC TTT GTA TCT AAT CAT TGC ATG TCT  815
 I   C   L   P   E   E   H   T   E   K   W   K   K   F   I   F   V   S   N   H   C   M   S   194

GAT GGT CGG TCT TCG ATC CAC TTT TTT CAT GAT TTA AGA GAC GAA TTA AAT AAT ATT AAA ACT CCA CCA  884
 D   G   R   S   S   I   H   F   F   H   D   L   R   D   E   L   N   N   I   K   T   P   P   217

AAA AAA TTA GAT TAC ATT TTC AAG TAC GAG GAG GAT TAC CAA TTG TTG AGG AAA CTT CCA GAA CCG ATC  953
 K   K   L   D   Y   I   F   K   Y   E   E   D   Y   Q   L   L   R   K   L   P   E   P   I   240

GAA AAG GTG ATA GAC TTT AGA CCA CCG TAC TTG TTT ATT CCG AAG TCA CTT CTT TCG GGT TTC ATC TAC 1022
 E   K   V   I   D   F   R   P   P   Y   L   F   I   P   K   S   L   L   S   G   F   I   Y   263

AAT CAT TTG AGA TTT TCT TCA AAA GGT GTC TGT ATG AGA ATG GAT GAT GTG GAA AAA ACC GAT GAT GTT 1091
 N   H   L   R   F   S   S   K   G   V   C   M   R   M   D   D   V   E   K   T   D   D   V   286

GTC ACC GAG ATC ATC AAT ATT TCA CCA ACA GAA TTT CAA GCG ATT AAA GCA AAT ATT AAA TCA AAT ATC 1160
 V   T   E   I   I   N   I   S   P   T   E   F   Q   A   I   K   A   N   I   K   S   N   I   309

CAA GGT AAG TGT ACT ATC ACT CCG TTT TTA CAT GTT TGT TGG TTT GTA TCT CTT CAT AAA TGG GGT AAA 1229
 Q   G   K   C   T   I   T   P   F   L   H   V   C   W   F   V   S   L   H   K   W   G   K   332

TTT TTC AAA CCA TTG AAC TTC GAA TGG CTT ACG GAT ATT TTT ATC CCC GCA GAT TGC CGC TCA CAA CTA 1298
 F   F   K   P   L   N   F   E   W   L   T   D   I   F   I   P   A   D   C   R   S   Q   L   355

CCA GAT GAT GAT GAA ATG AGA CAG ATG TAC AGA TAT GGC GCT AAC GTT GGA TTT ATT GAC TTC ACC CCA 1367
 P   D   D   D   E   M   R   Q   M   Y   R   Y   G   A   N   V   G   F   I   D   F   T   P   378

TGG ATA AGC GAA TTT GAC ATG AAT GAT AAC AAA GAA AAA TTT TGG CCA CTT ATT GAG CAC TAC CAT GAA 1436
 W   I   S   E   F   D   M   N   D   N   K   E   K   F   W   P   L   I   E   H   Y   H   E   401

GTA ATT TCG GAA GCT TTA AGA AAT AAA AAG CAC CTC CAT GGC TTA GGG TTC AAT ATA CAA GGC TTC GTT 1505
 V   I   S   E   A   L   R   N   K   K   H   L   H   G   L   G   F   N   I   Q   G   F   V   424

CAA AAA TAT GTG AAT ATT GAC AAG GTA ATG TGC GAT CGT GCC ATC GGG AAA AGA CGC GGA GGT ACA TTG 1574
 Q   K   Y   V   N   I   D   K   V   M   C   D   R   A   I   G   K   R   R   G   G   T   L   447

TTA AGC AAT GTA GGT CTG TTT AAT CAG TTA GAG GAG CCC GAT GCC AAA TAT TCT ATA TGC GAT TTG GCA 1643
 L   S   N   V   G   L   F   N   Q   L   E   E   P   D   A   K   Y   S   I   C   D   L   A   470

TTT GGC CAA TTT CAA GGA TCC TGG CAC CAA GCA TTT TCC TTG GGT GTT TGT TCG ACT AAT GTA AAG GGG 1712
 F   G   Q   F   Q   G   S   W   H   Q   A   F   S   L   G   V   C   S   T   N   V   K   G   493

ATG AAT ATT GTT GTT GCT TCA ACA AAA AAT GTT GTT GGT AGC CAA GAA TCT CTC GAA GAG CTT TGC TCC 1781
 M   N   I   V   V   A   S   T   K   N   V   V   G   S   Q   E   S   L   E   E   L   C   S   516

ATT TAT AAA GCT CTC CTT TTA GGC CCT TAG ATCTCACATGATGCTTGACTGATATTATTCGACAATATGATTATGTCGTGT 1862
 I   Y   K   A   L   L   L   G   P   *                                                        525

AAATAACCCACTTTCATGTTGTCACTCCCTCGGCTTTGGTTGGTTAAAGGGACTTATTGGT                               1923
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FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *ATF1* gene from *S. cerevisiae* Kyokai No. 7. The amino acids confirmed by peptide sequencing are underlined. The putative TATA-like sequence and additional poly(A) signal are indicated by wavy underlining.

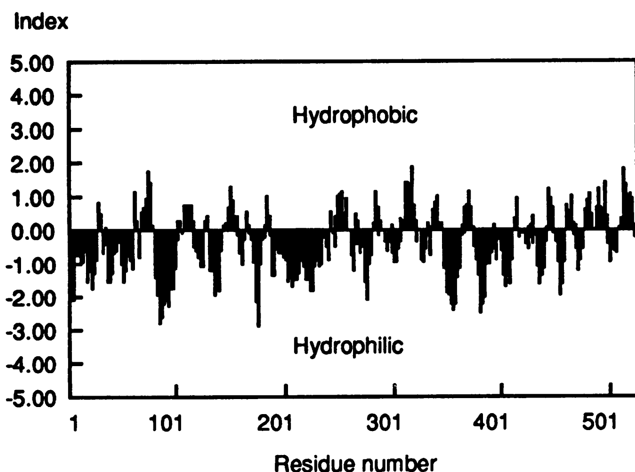

FIG. 4. Hydrophobicity analysis of the Atfl protein. The Kyte-Doolittle plot was generated by using groups of 10 residues.

sis. The most obvious regulator of AATase activity is oxygen. The AATase activity of yeast cells and the acetate ester concentrations in media obviously decrease under aerobic conditions (20, 23). It has been suggested that AATase activity is inhibited by unsaturated fatty acids (22) and that the concentration of unsaturated fatty acids in the cell membrane affects AATase activity (23). Recently, Malcorps et al. (10) proposed that gene repression is the main cause of decreases in AATase activity in the presence of oxygen and unsaturated fatty acids. It was necessary to clone the AATase-encoding gene in order to understand the mechanism of ester formation.

Because there is no simple method to measure AATase activity on plates, enzyme purification was a necessary step in cloning the AATase gene. The 6.6-kb *Xba*I fragment that was cloned from *S. cerevisiae* was shown to encode the *ATF1* gene.

The nucleotide sequence of the *ATF1* gene revealed that this gene encodes a protein with a molecular weight of 61,059. This value is consistent with the predicted molecular weight of the purified AATase (60,000). All 10 peptide sequences which were determined by sequencing the purified AATase (Fig. 1) are present in this predicted protein (Fig. 3). When yeast cells were transformed with the *ATF1* gene carried on a multicopy plasmid, the resulting transformants exhibited high levels of AATase activity in static cultures. These results strongly suggest that the Atfl protein is the same as AATase.

The results of the *ATF1* gene and Atfl protein sequence analysis revealed some well-known features of AATase. First, the codon usage data suggested that the Atfl protein has a low codon bias index value (0.07), as defined by Bennetzen and Hall (2). It has been found that the codon bias index values of highly expressed yeast glycolytic genes, such as *ENO2*, *PGK1*, and *PYK*, are 0.96 to 0.91 and that the codon bias index values of rarely expressed yeast regulatory genes, such as *GAL4*, *GAL80*, *MATa1*, *RAS1*, and *RAS2*, are 0.04, 0.08, 0.05, 0.17, and 0.22, respectively. The low codon bias index value of the *ATF1* gene is consistent with the low level of expression of AATase.

TABLE 1. AATase specific activities of transformants

Strain/plasmid	AATase sp act (μmol of isoamyl acetate min^{-1} mg of $protein^{-1}$)
TD4/YEp13k	1.5 (0.4)[a]
TD4/YATK11(*ATF1*)[b]	15.9
TD4/YATL1(*ATF1*)[c]	22.0 (0.4)
TD4/YATL2(Lg-*ATF1*)[c]	9.6 (0.6)

[a] The values in parentheses are the AATase specific activities in shaken cultures.
[b] The gene originated from *S. cerevisiae* K7.
[c] The gene originated from brewery lager yeast.

TABLE 2. Volatile ester concentrations in cell culture supernatants

Strain/Plasmid	Concn of:					
	Ethanol (% wt/wt)	Ethyl acetate (ppm)	Isoamyl alcohol (ppm)	Isoamyl acetate (ppm)	Isobutyl alcohol (ppm)	*n*-Propanol (ppm)
TD4/YEp13k	1.9	3.0	22.6	0.1	13.7	6.0
TD4/YATL1 (*ATF1*)[a]	1.6	27.1	21.5	2.7	9.8	6.9
TD4/YATL2 (Lg-*ATF1*)[a]	1.7	6.0	20.0	0.7	8.5	6.0

[a] The gene originated from brewery lager yeast.

Second, the Atfl protein contains 14 cysteine residues out of 525 amino acids. Thus, the proportion of cysteine residues in this protein is greater than the proportion of cysteine residues in other known acetyltransferases and acyltransferases. AATase is known to be highly labile, and it is possible that the high proportion of cysteine residues is responsible in part for the lability of this enzyme.

The sequence analysis also revealed a unexpected feature of AATase. Although AATase has been shown previously to be a membrane-bound enzyme (10, 12, 13, 22, 23), a hydrophobicity analysis of the Atfl protein revealed that this protein is not hydrophobic. Although there are some short hydrophobic segments which could possibly interact with membranes, the sequencing results suggested that the Atfl protein tends to be hydrophilic rather than hydrophobic and that potentially hydrophobic transmembrane segments are absent (Fig. 4). The Atfl protein has no processed segment in the N-terminal region to act as a potential signal sequence for secretion or as an intracellular targeting segment, as do proteins destined for the mitochondria and endoplasmic reticulum. Malcorps and Dufour proposed that the enzyme might be loosely bound to the vacuole (11). Our results suggest that the Atfl protein is not an integral membrane protein but is a membrane-associated protein.

The results of the computer analysis showed that there was no extensive sequence similarity between the translated amino acid sequence of the Atfl protein and any other known protein-encoding sequence in the GenBank, EMBL, NBRF-PIR, and SWISS-PROT databases. However, by comparing the Atfl protein with previously described acetyltransferases and acyltransferases, we found that the Atfl protein has a short sequence in the C-terminal region that is homologous to the *HEM0* and *HEM1* gene products of humans, chickens, and mice (3). These genes encode 5-aminolevulinic acid synthase (EC 2.3.1.37), which catalyzes the synthesis of aminolevulinate from succinyl coenzyme A and glycine. A sequence of five amino acids in the C-terminal region of the Atfl protein (LEELC, positions 511 to 515 in Fig. 3). The function of this homologous domain is unknown, but both AATase and 5-aminolevulinic acid synthase are inhibited by sulfhydryl reagents and are membrane-bound proteins. Interestingly, the Hem0 and Hem1 proteins also contain numerous cysteine residues (1.9 to 2.6% of the total residues).

Expression of the *ATF1* and Lg-*ATF1* genes in yeast cells. The results of a Southern analysis of yeast genome DNAs suggested that *S. cerevisiae* has a unique *ATF1* gene, while brewery lager yeast has one *ATF1* gene and another, homologous gene (the Lg-*ATF1* gene).

Transformants which had the *ATF1* gene or the Lg-*ATF1* gene exhibited high levels of AATase activity in static cultures. This finding suggested that the Lg-*ATF1* gene encodes AATase or its activator. We speculated that this homologous gene might be a derivative of the *ATF1* gene, because one of the major characteristics of brewery lager yeast is that it is an allopolyploid which has at least two diverged genomes. Functionally similar but structurally different (or homologous) alleles have been reported for many other genes, including *ERG10* (4), *LEU2* (14), *MET2* (6), *URA3*, *CYC7*, *HIS4*, and *MAT* (7). To confirm that the Lg-*ATF1* gene is a derivative of the *ATF1* gene, sequence data will be necessary.

The results of a comparison of the *ATF1* gene and the Lg-*ATF1* gene suggested that the levels of AATase activity produced are different for the *ATF1* gene and the Lg-*ATF1* gene. The *ATF1* gene transformants exhibited greater AATase activities (Table 1) and produced greater concentrations of esters than the Lg-*ATF1* gene transformants (Table 2). We confirmed by Southern analysis that the differences in the copy numbers of plasmids for three transformants were small (less than 10%) under the culture conditions used (data not shown). It is not clear at present whether the differences in AATase activity result from differences in the levels of gene expression or are due to differences in the specific activities of the gene products. Investigations in which Northern (RNA) analysis is used should clarify this issue.

***ATF1* gene plays a key role in ethyl acetate and isoamyl acetate synthesis.** The results of an analysis of ester production by the transformants clearly demonstrated that the *ATF1* gene is useful for the control of ester production. The concentrations of both ethyl acetate and isoamyl acetate in the culture supernatants increased compared with the parent strain, and these increases depended on the levels of AATase activity of the transformants, while the production of ethanol and other higher alcohols did not change.

Ashida et al. isolated a mutant which produces high concentrations of isoamyl alcohol and isoamyl acetate during fermentation (1). These authors suggested that the level of isoamyl alcohol production is a major limiting factor in isoamyl acetate production during fermentation. Our data strongly suggest that AATase activity is also a major limiting factor in isoamyl acetate and ethyl acetate production.

However, it should be noted that the increases in production of ethyl acetate and isoamyl acetate are not identical. It was observed that for either the *ATF1* gene or the Lg-*ATF1* gene the increase in isoamyl acetate concentration was greater than the increase in ethyl acetate concentration, compared with the control.

This difference in the ratio of increases may be due to AATase substrate specificity, since it has been suggested that AATase has a greater affinity for isoamyl alcohol than for ethanol (13, 22).

Recently, one other type of acetyltransferase has been reported to be responsible for the production of ethyl acetate in yeast cells (10). Our data strongly suggest that the Atf1 protein plays a key role in both isoamyl acetate synthesis and ethyl acetate synthesis. However, in our preliminary experiment, an *atf1::URA3* strain still exhibited AATase activity which was 5 to 10 times lower than the activity of the control. It is possible that yeast cells contain many different types of AATase and that this makes it difficult for brewers to control ester production. In order to evaluate the precise role of the *ATF1* gene in isoamyl acetate and ethyl acetate synthesis during fermentation, further studies are necessary.

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