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

TOSHIRO FUJII,1* NAOSHI NAGASAWA,2 AKIHIRO IWAMATSU,1 TAKAYUKI BOGAKI,2
YUKIO TAMAI,1 AND MASAAKI HAMACHI2

Central Laboratories for Key Technology, Kirin Brewery Co., Ltd., 1-13-5, Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa 236,1 and General Research Laboratory, Ozeki Corp., Nishinomiya-shi, Hyogo 663,2 Japan

Received 24 January 1994/Accepted 15 May 1994

The ATF gene, which encodes alcohol acetyltransferase (AATase), was cloned from Saccharomyces cerevisiae and brewery lager yeast (Saccharomyces uvarum). The nucleotide sequence of the ATF 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 ATF gene was used as a probe revealed that S. cerevisiae has one ATF gene, while brewery lager yeast has one ATF gene and another, homologous gene (Lg-ATF1). Transformants carrying multiple copies of the ATF gene or the Lg-ATF1 gene exhibited high AATase activity in static cultures and produced greater concentrations of acetal esters than the control.

Acetate esters, such as isoamy1 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 ATF 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 ATF gene, but brewery lager yeast has one ATF gene and another homologous gene. We also obtained expression of these genes by using a multicyclic 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.

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 his-t19 ura3-52 leu2-3 leu2-112 trp can) was used for transformation and for expression of the ATF1 monocopy plasmid. Escherichia coli DH5 (endA1 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 Sau3AI to give fragments with an average size of 15 to 20 kb. These fragments were ligated into the BamHI site of the λ-EMBL3 vector (Stratagene) and packaged in vitro (Stratagene). E. coli P2392 {F− galK2 galT22 hsdR514 [F− m4] λ− lacY1 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-32P]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

* Corresponding author. Phone: 81-45-788-7200. Fax: 81-45-788-4042.
in a solution containing 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.4]), 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 ClaI-EcoRI fragment of the S. cerevisiae ATF1 gene was used as a probe after labelling with [α-³²P]dCTP using a multimix 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 (Δlac-proAB) endA1 gyrA96 hsdR17 thi λ relA1 supE44 [F' traD36 proAB lacI² ZAM15]) and bacteriophage M13mp18,mp19 (21) were used to clone DNA fragments for sequencing. Sequencing was performed by the dyeode chain termination method (18), using a Bsr 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% isomycol 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 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 EcoRI-BamHI fragment in Fig. 2a.

**Nucleotide sequence of the ATF1 Gene.** The 6.6-kb XbaI 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 ClaI or XbaI when the 0.4-kb ClaI-EcoRI 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.
We then cloned the AATase-encoding genes from the \( \lambda \)-EMBL3 library constructed from KBY001 DNA by the 0.4-kb ClaI-EcoRI fragment from the \( S. \) cerevisiae Kyokai No. 7 \( ATF1 \) gene as the probe. We screened a total of 30,000 recombinants from the \( \lambda \)-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 \( XbaI \) fragment from \( S. \) cerevisiae Kyokai No. 7 and the 6.6-kb \( XbaI \) fragment and 5.7-kb BglII 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 acetyl ester production in yeast cells. AATase is recognized as an enzyme that plays a primary role in acetyl 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 isomyal 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-
FIG. 3. Nucleotide sequence and deduced amino acid sequence of the \textit{ATF1} gene from \textit{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.
sis. The most obvious regulator of AATase activity is oxygen. The AATase activity of yeast cells and the acetate ester concentrations in media obviously 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 XbaI 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 Benetzen and Hall (2). It has been found that the codon bias index values of highly expressed yeast glycolytic genes, such as ENO2, PGK1, and PK, are 0.96 to 0.91 and that the codon bias index values of rarely expressed yeast regulatory genes, such as GAL4, GAL80, MAP1, 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.

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 acyltransferases and acyltransferases. AATase is known to be highly labile, and it is possible that the high proportion of cysteine residues is responsible for part of the lability of this enzyme.

The sequence analysis also revealed an 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 of sequence analysis 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-PRF, and SWISS-PROT databases. However, comparing the Atfl protein with previously described acyltransferases 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-amino-levulinate 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).

| Strain/Plasmid | Ethanol (% wt/wt) | Ethyl acetate (ppm) | Isomyl alcohol (ppm) | Isoybutyl alcohol (ppm) | n-Propanol (ppm) |
|---------------|------------------|---------------------|----------------------|------------------------|------------------|
| TD4/YEp13k    | 1.9              | 3.0                 | 22.6                 | 0.1                    | 13.7             | 6.0              |
| TD4/YATK11(ATF1)* | 1.6             | 27.1                | 21.5                 | 2.7                    | 9.8              | 6.9              |
| TD4/YATL1(ATF1)* | 1.7             | 6.0                 | 20.0                 | 0.7                    | 8.5              | 6.0              |
| TD4/YATL2(Lg-ATF1)* | 9.6            | 9.0                 |                     |                        |                  |

* The gene originated from brewery lager yeast.

* The gene originated from S. cerevisiae K7.

* The gene originated from brewery lager yeast.

FIG. 4. Hydrophobicity analysis of the Atfl protein. The Kyte-Doolittle plot was generated by using groups of 10 residues.
Expression of the ATFI and Lg-ATFI genes in yeast cells.

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

Transformants which had the ATFI gene or the Lg-ATFI gene exhibited high levels of AATase activity in static cultures. This finding suggested that the Lg-ATFI gene encodes AATase or its activator. We speculated that this homologous gene might be a derivative of the ATFI 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 MAT7 (7). To confirm that the Lg-ATFl gene is a derivative of the ATFI gene, sequence data will be necessary.

The results of a comparison of the ATFI gene and the Lg-ATFI gene suggested that the levels of AATase activity produced are different for the ATFI gene and the Lg-ATFI gene. The ATFI gene transformants exhibited greater AATase activities (Table 1) and produced greater concentrations of esters than the Lg-ATFI 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. ATFI 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 ATFI 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 ATFI gene or the Lg-ATFI 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 ATfl protein plays a key role in both isoamyl acetate synthesis and ethyl acetate synthesis. However, in our preliminary experiment, an atfi::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 ATFI gene in isoamyl acetate and ethyl acetate synthesis during fermentation, further studies are necessary.

ACKNOWLEDGMENTS

We thank Yataro Nunokawa, Jun-ichi Tanaka, and Kazuo Yoshioka for their helpful comments and Y. Sawano for his technical assistance in DNA sequencing.

REFERENCES

1. Ashida, S., E. Ichikawa, K. Sugimami, and S. Imayasu. 1987. Isolation and application of mutants producing sufficient isoamyl acetate, a sake flavor component. Agric. Biol. Chem. 51:2061–2065.
2. Bennetzen, J. L., and B. D. Hall. 1978. Codon selection in the yeast Saccharomyces cerevisiae. J. Biol. Chem. 253:3026–3031.
3. Cox, T. C., M. J. Bawden, A. Martin, and B. K. May. 1991. Human erythroid 5-aminolevulinate synthase: promoter analysis and identification of an iron-response element in the mRNA. EMBO J. 10:1891–1902.
4. Dequin, S., R. Gloclecker, C. J. Herbert, and F. Boutelet. 1988. Cloning, sequencing and analysis of the yeast S. uvarum ERG10 gene encoding acetoacetyl CoA thiolase. Curur. Genet. 13:471–478.
5. Hanahan, D. 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166:557–580.
6. Hansen, J., and M. C. Killand-Brandt. 1994. Saccharomyces carlsbergensis contains two functional MET2 alleles similar to homologues from S. cerevisiae and S. monacensis. Gene 140:33–40.
7. Holmberg, S. 1982. Genetic differences between Saccharomyces carlsbergensis and S. cerevisiae. II. Restriction endonuclease analysis of genes in chromosome III. Carlsberg Res. Commun. 47:223–244.
8. Ito, H., K. Murata, and A. Kimura. 1984. Transformation of intact yeast cells treated with alkali cations or thiol compounds. Agric. Biol. Chem. 48:341–347.
9. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mbl Biol. 157:105–132.
10. Malcorps, P., J. M. Cheval, S. Jamil, and J. P. Dufour. 1991. A new model for the regulation of ester synthesis by alcohol acetyltransferase in Saccharomyces cerevisiae during fermentation. J. Am. Soc. Brew. Chem. 49:47–53.
11. Malcorps, P., and J. P. Dufour. 1987. Ester synthesis by Saccharomyces carlsbergensis localization of the acetyl-CoA:isoamyl alcohol acetyl transferase ("AT"). Eur. Brew. Conv. 21:377–384.
12. Malcorps, P., and J. P. Dufour. 1992. Short-chain and medium chain aliphatic-ester synthesis in Saccharomyces cerevisiae. Eur. J. Biochem. 210:1015–1022.
13. Minekata, T., T. Bogaki, A. Iwamatsu, T. Fujii, and M. Hamachi. 1993. The purification, properties and internal peptide sequences of alcohol acetyltransferase from Saccharomyces cerevisiae Kyokai No. 7. Biosci. Biotechnol. Biochem. 57:2094–2098.
14. Pedersen, M. B. 1985. DNA sequence polymorphisms in the genus Saccharomyces. IV. Homoeologous chromosomes III of Saccharomyces bayanus, S. carlsbergensis, and S. uvarum. Carlsberg Res. Commun. 180:263–272.
15. Rice, J. F., E. Chicoje, R. Helbert, and J. Graver. 1977. Inhibition of beer volatiles formation by carbon dioxide pressure. J. Am. Soc. Brew. Chem. 35:350.
16. Rose, M. D., and J. R. Broach. 1991. Cloning genes by complementation in yeast. Methods Enzymol. 194:195–229.
17. Rose, M. D., F. Winston, and P. Hieter. 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
19. Sone, H., T. Fujii, K. Kondo, F. Shimizu, T. Tanaka, and T. Inoue. 1988. Nucleotide sequence and expression of the Enterobacter aerogenes α-acetolactate decarboxylase gene in brewers’ yeast.
20. Wilson, R. J. H. 1978. The influence of oxygen and fermentation and storage. Eur. Brew. Conv. Symp. 5:17–27.

21. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M-13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33:103–119.

22. Yoshioka, K., and H. Hashimoto. 1981. Ester formation by alcohol acetyltransferase from brewers' yeast. Agric. Biol. Chem. 45:2183–2190.

23. Yoshioka, K., and H. Hashimoto. 1983. Cellular fatty acid and ester formation by brewers' yeast. Agric. Biol. Chem. 47:2287–2294.