Appl Microbiol Biotechnol (2002) 59:501–508
DOI 10.1007/s00253-002-1041-5

ORIGINAL PAPER

H. Yoshimoto · T. Fukushige · T. Yonezawa · H. Sone

Genetic and physiological analysis of branched-chain alcohols and isoamyl acetate production in *Saccharomyces cerevisiae*

Received: 12 February 2002 / Revised: 24 April 2002 / Accepted: 3 May 2002 / Published online: 22 June 2002

Abstract Branched-chain alcohols, such as isoamyl alcohol and isobutanol, and isoamyl acetate are important flavor components of yeast-fermented alcoholic beverages. Analysis of a null mutant of the *BAT2* gene encoding cytosolic branched-chain amino acid aminotransferase, and a transformant with multi-copy plasmids containing the *BAT2* gene showed that the *BAT2* gene product plays an important role in the production of branched-chain alcohols and isoamyl acetate. Fermentation tests using the *bat2* null mutant transformed with multi-copy plasmids carrying the *ATF1* gene, which encodes alcohol acetyltransferase, indicated that modified expression of *BAT2* and *ATF1* genes could significantly alter the proportion of branched-chain alcohols and isoamyl acetate synthesized. Furthermore, fermentation tests using different ratios of nitrogen source and RNA blot analyses demonstrated that transcription of L-leucine biosynthetic (*LEU*) and *BAT* genes is co-regulated by nitrogen source, that production of isoamyl alcohol depends on this transcription, and that *ATF* transcription increased with increased concentrations of nitrogen source. Our data suggest that changes in isoamyl alcohol production by nitrogen source are due to transcriptional co-regulation of *LEU* and *BAT* genes, and that production of isoamyl acetate is dependent on isoamyl alcohol production and *ATF* transcription.

Introduction

Flavor is one of the most important factors in the quality of yeast-fermented alcoholic beverages. Branched-chain alcohols, such as isoamyl alcohol and isobutanol, and isoamyl acetate are produced during fermentation and are an important contributor to the flavor.

The branched-chain alcohols are synthesized from the corresponding intermediates – α-keto-acids – in the branched-chain amino acids (L-leucine and L-valine) metabolic pathway, by decarboxylation and reduction (Dickinson et al. 1997; Iersel et al. 1997; Singh and Kunkee 1976; Yoshimoto et al. 2001). These α-keto acids are formed via two major pathways: catabolic [also known as the Ehrlich pathway (Rainbow 1970)] and anabolic pathways involved in de novo synthesis of branched-chain amino acids through their biosynthetic pathway from glucose. The first step in catabolism of branched-chain amino acids is transamination to form their respective α-keto acids (Dickinson and Norte 1993; Ichihara and Koyama 1966). This reaction is catalyzed by mitochondrial and cytosolic branched-chain amino acid aminotransferases encoded by the *BAT1* and *BAT2* genes, respectively. These genes have been isolated and analyzed (Eden et al. 1996, 2001; Kispal et al. 1996).

Isoamyl acetate is synthesized from acetyl CoA and isoamyl alcohol by alcohol acetyltransferases encoded by the *ATF1* (Fujii et al. 1994, 1997; Fujiwara et al. 1998, 1999; Yoshimoto et al. 1998) and *ATF2* genes (Nagasawa et al. 1998; Yoshimoto et al. 1999). It was shown that transformants carrying the *ATF1* gene on a multi-copy plasmid exhibited a 27-fold increase in isoamyl acetate production (Fujii et al. 1994). Several approaches have been reported to control the production of branched-chain alcohols and isoamyl acetate (Ashida et al. 1987; Hirata and Hiroi 1991; Watanabe et al. 1990). However, mutants that produce a low amount of isoamyl alcohol and a high amount of isoamyl acetate have not yet been isolated.

The influence of nitrogen source on production of branched-chain alcohols has been reported (Hammond 1993; Oshita et al. 1995; Rainbow 1970). At low levels of assimilable nitrogen source, the biosynthetic pathway predominates whereas at high level, the Ehrlich pathway becomes prominent as a result of feedback and/or repression of key enzymes in the biosynthetic pathway. How-

H. Yoshimoto (✉) · H. Sone
Central Laboratories for Key Technology, Kirin Brewery Co. Ltd.,
1–13–5, Fukuura, Kanazawa-ku, Yokohama-shi,
Kanagawa 236–0004, Japan
e-mail: hyoshimoto@kirin.co.jp
Tel.: +81-45-7887200, Fax: +81-45-7884041

T. Fukushige · T. Yonezawa
Gotemba Plant, Kirin-Seagram Limited, 970, Shibanuta,
Gotemba, Shizuoka 412–0003, Japan

ever, transcriptional regulation by nitrogen source has not been investigated well in either of these pathways.

The aim of this study is to investigate the mechanism of branched-chain alcohol and isoamyl acetate production. We examined the role of the *BAT2* gene in the production of the two types of compound, and whether modified *BAT2* and *ATF1* expression results in production of a diversity of flavor components. We also examined the effects of nitrogen source on the production and on transcriptional regulation.

Materials and methods

Strains and media

The yeast strains used in this study are listed in Table 1. YPD10 medium (1% yeast extract, 2% peptone, 10% glucose) was used to cultivate yeast strains. A synthetic medium containing various concentrations of the nitrogen source was used to investigate the effect of nitrogen source. The composition of this medium was based on the study of Oshita et al. (1995), and consisted of the following: sugar source (% w/v): glucose 10.0; nitrogen source (mM): amino acids 13.4, ammonium citrate 14.0; vitamins (mg/l): thiamine hydrochloride 0.6, riboflavin 0.4, pyridoxine hydrochloride 0.85, nicotinamide 12.0, calcium pantothenate 0.5, biotin 0.0065, folic acid 0.04, *p*-aminobenzoic acid 1.0, inositol 100.0; salts (mg/l): KH_2PO_4 2,200, KCl 1,700, $CaCl_2 \cdot 2H_2O$ 250, $MgSO_4 \cdot 7H_2O$ 500, $Fe_2(SO_4)_3 \cdot H_2O$ 10, $MnSO_4 \cdot H_2O$ 10, $ZnSO_4 \cdot 7H_2O$ 10, $CuSO_4 \cdot 5H_2O$ 1, H_3BO_4 0.1, $Na_2MoO_4 \cdot 2H_2O$ 0.1; buffer (g/l): potassium citrate 4.0, citric acid 0.8. Fermentation trials were carried out using five types of synthetic medium containing 0, 0.5, 1, 2, and 3-fold amounts of nitrogen source compared to the amount given above.

Culture conditions

Null mutants and transformants were precultured with shaking at 30°C for 2 days in 100 ml of YPD10 medium containing 0.3 mg/ml G418 to select the maintenance of plasmids. The cells were harvested and diluted to a density of 5×10^5 cells/ml, and then grown at 30°C under anaerobic conditions produced by initial headspace exclusion and N_2 flushing, stirring gently with a magnetic stir-bar in 250 ml of the same medium. To study the effect of nitrogen source, fermentation tests of YHY554 cells, which do not have any auxotrophic requirements, were performed using synthetic medium containing various concentrations of the nitrogen source. Cells were precultured with shaking at 30°C for 2 days in the synthetic medium containing a 1-fold amount of nitrogen source, harvested and diluted to a density of 5×10^5 cells/ml, and grown at 30°C under anaerobic conditions as described above in five types of synthetic medium containing 0, 0.5, 1, 2, and 3-fold amounts of nitrogen source.

Analysis of branched-chain alcohols and acetate esters

Branched-chain alcohols and acetate esters were analyzed by headspace gas chromatography (Shimadzu HSS-4A head space analysis system). These were separated using a Megabore DB-WAX column (0.53 mm i.d. ×30 m) after autoinjection of the headspace sample. The following conditions were also applied: injection temperature 200°C; oven temperature 40°C for 5 min and 140°C for 3 min; detector temperature 200°C; and carrier gas N_2.

Disruption of the *BAT2* gene

The *bat2::LEU2* deletion cassette was constructed by polymerase chain reaction (PCR) using a two-step procedure (Amberg et al. 1995; Wach 1996). The oligonucleotides used as primers are listed in Table 2. First, the 0.6-kb upstream long flanking homologous (LFH) region and the 0.4-kb downstream LFH region were constructed using chromosomal DNA obtained from strain KY1055 as template with primers P150 and P152, and P151 and P153, respectively. A 1.4-kb DNA fragment containing the *URA3* gene was amplified by P53 and P54 using plasmid YEp24 (Botstein et al. 1979) as template. In the second step, the *bat2::LEU2* deletion cassette was amplified using P154 and P155 in the presence of upstream and downstream LFH regions, and a DNA fragment containing the *URA3* gene as template. This reaction generated the gene-specific deletion cassettes. The resulting 2.2-kb fragment was used to transform strain KY1055 to generate strain KY1056. Replacement of the chromosomal *BAT2* allele was confirmed by PCR. To prepare the control strain, strain KY1055 was transformed with the 1.4-kb DNA fragment containing the *URA3* gene to generate KY1057. Since the fermentation test was performed under the same conditions, strains KY1056 and KY1057 were transformed with the multi-copy plasmid vector pYT77 carrying the G418 resistance gene (Tamai 1996) to generate strains KY1058 and KY1059, respectively.

Overexpression of *BAT2* and *ATF1* genes

The 2.0-kb DNA fragment containing the *BAT2* gene was amplified by P150 and P155 using chromosomal DNA obtained from strain KY1055 as template and directly cloned into the *Eco*RV site of vector pT7/BlueT (Novagen) to yield the plasmid pHY490. This plasmid was digested with *Bam*HI and *Sal*I. The *Bam*HI and *Sal*I fragments containing the *BAT2* gene, were cloned into the *Bam*HI and *Hin*dIII sites of the yeast expression vector plasmid pYT77 (Tamai 1996) to yield the plasmid pHY492. Strain KY1057 was transformed with the resulting plasmid (pHY492) to generate strain KY1060. Strain KY1056 was also transformed with the multi-copy plasmid p*ATF1*/77 (Tamai 1996), containing the *ATF1* gene, to generate strain KY1061.

Table 1 Strains used in this work

Strain	Genotype (plasmid)	Source
TD4	*MATa his4–519 ura3–52 leu2–3 leu2–112 trp1 can*^{r}	Fujii et al. 1994
YHY554	*MATa*	Our stock culture
KY1055[a]	*MATa BAT1 BAT2 LEU2 ura3–52 his4–519 trp1 can*^{r}	This work
KY1056	*MATa BAT1 bat2::URA3 LEU2 his4–519 trp1 can*^{r}	This work
KY1057	*MATa BAT1 BAT2 LEU2 URA3 his4–519 trp1 can*^{r}	This work
KY1058	*MATa BAT1 bat2::URA3 LEU2 his4–519 trp1 can*^{r} (vector: pYT77)	This work
KY1059	*MATa BAT1 BAT2 LEU2 URA3 his4–519 trp1 can*^{r} (vector: pYT77)	This work
KY1060	*MATa BAT1 BAT2 LEU2 his4–519 trp1 can*^{r} (p*BAT2*: pHY492)	This work
KY1061	*MATa BAT1 bat2::URA3 LEU2 his4–519 trp1 can*^{r} (p*ATF1*: p*ATF1*/77)	This work

[a] Strain TD4 transformed with DNA fragments of *LEU2* gene amplified by PCR

Table 2 Oligonucleotides used in this work

Name	Sequence	Nucleotide position[a]
For *ATF1*		
P142	5′-AAGGTTAGGGTTTATGGACCCAGGCAGCAG-3′	–380
P143	5′-GTATATACCAGCTCCCACAGGACGACGATT-3′	+1,900
For *ATF2*		
P113	5′-AATCAGTATTTCAATCGCCACAATCTCAGG-3′	–330
P114	5′-TGCGAGGTAAGACTATTGTCTAAGACTTAT-3′	+1,770
For *BAT1*		
P160	5′-CCTTCTGCACTTCTCAAATTGATAGCATTG-3′	–470
P161	5′-ACTGTACTTGCACCTCTTCTGAACTACGCT-3′	+1,600
For *BAT2*		
P150	5′-CAACTTATAACGCTCCTTTCCAAACATCTT-3′	–510
P151	5′-TTAAACATTTTTGGAAAGATCTGCTCATAT-3′	+1,510
P152[b]	5′-CCGCTCAGGTCCTTGTCCTTTAACGAGGCCTTATCTTAACTTTGGAGGCGTCTAGGGGTG-3′	+40
P153[b]	5′-GCGCTCATCGTCATCCTCGGCACCGTCACCAGCATGGCAATTGGTCAAGGGTTGTTACTG-3′	+1,091
P154	5′-CCGCACTACACCAAAGTTTAATGTTCCATT-3′	–400
P155	5′-ATTACGGAAACGTCTTGAAATATTAGTTGG-3′	+1,460
For *KID1*		
P210	5′-ATATGCAGAACCTTGGCAACTGTAAGAACT-3′	–280
P211	5′-CAAAGTTGCGTGGTATCATCTTACGATATT	+2,160
For *LEU1*		
P178	5′-ATGGTTTACACTCCATCCAAGGGTCCAAGA-3′	+1
P179	5′-CTACCAATCCTGGTGGACTTTATCGAAAGT-3′	+2,340
For *LEU4*		
P156	5′-ATGGTTAAAGAGAGTATTATTGCTCTTGCT-3′	+1
P157	5′-TTATGCAGAGCCAGATGCCGCAGCATTCTT-3′	+1,860
For *PDC1*		
P107	5′-ACAAGCTCATGCAAAGAGGTGGTACCCGCA-3′	–895
P108	5′-TGGAAACCACACTGTTTAAACAGTGTTCCT-3′	+2,015
For *URA3*		
P53	5′-GGCCTCGTTAAAGGACAAGGACCTGAGCGG-3′	–338
P54	5′-GGTGACGGTGCCGAGGATGACGATGAGCGC-3′	+1,041

[a] The nucleotide position refers to the nucleotide in the gene target sequence corresponding to the first base at the 5′-end of the oligonucleotide. The first base of the target ORF (A of ATG codon) is designated +1

[b] The sequence complementary to the *URA3* gene is underlined

RNA blot analysis

Total RNA was extracted from YHY554 cells harvested at the same growth stage (OD_{600}) during the exponential-phase by the glass-beads method (Elion and Warner 1984), separated on a 1% agarose gel, transferred to nylon membrane and subjected to RNA blot analysis (Yamashita and Fukui 1985). The fragments of *ATF1*, *ATF2*, *BAT1*, *BAT2*, *KID1*, *LEU1*, *LEU2*, *LEU4*, and *PDC1* genes lie on open reading frames and were obtained through PCR amplification with the primer pairs described in Table 2. The 1.8-kb *Hpa*I-*Sal*I fragment of YEp13 (Broach et al. 1979) was used as a probe for the *LEU2* gene. Each probe was generated by random primed labeling of fragments of each gene with (α-^{32}P) dCTP and a Multiprime DNA labeling kit (Amersham). To minimize the cross hybridization between *ATF1* and *ATF2*, *BAT1* and *BAT2*, or *PDC1* and *KID1*, the membrane was washed twice with 2× SSC containing 0.1% SDS at room temperature for 10 min and then twice more with 0.1× SSC containing 0.1% SDS at 65°C for 15 min.

Results

The *BAT2* gene product influences production of branched-chain alcohols and isoamyl acetate

To understand the role of cytosolic branched-chain amino acid aminotransferase in the production of branched-chain alcohols and isoamyl acetate, we constructed a null mutant (KY1058) for the *BAT2* gene and a transformant (KY1060) with multi-copy plasmids containing the *BAT2* gene. The concentrations of branched-chain alcohols and isoamyl acetate in the culture supernatants were determined following cultivation of these strains at 30°C for 4 days in YDP10 medium, which contained 0.3 mg/ml G418 for selective maintenance of the plasmids (Table 3). The growth of the *bat2* null mutant KY1058 was not impaired under these conditions (data

Table 3 Production of higher alcohols and acetate esters. *i-AmOH* Isoamyl alcohol, *i-BuOH* isobutanol, *n-PrOH n*-propanol, *IsOAc* isoamyl acetate, *EtOAc* ethyl acetate

Strain	Genotype (plasmid)	Production[a] [mg/l (%)]				
		i-AmOH	i-BuOH	n-PrOH	IsOAc	EtOAc
KY1059	*BAT1 BAT2 LEU2 URA3* (vector)	173.0±7.3(100)	69.3±5.5(100)	32.1±4.2(100)	4.3±0.05(100)	25.3±2.8(100)
KY1058	*BAT1 bat2::URA3 LEU2* (vector)	103.8±6.8(60)	19.3±3.5(28)	31.2±5.2(97)	1.8±0.02(42)	22.0±3.3(87)
KY1060	*BAT1 BAT2 LEU2 URA3* (multi-copy *BAT2*)	226.2±9.2(131)	154.0±7.6(222)	30.3±4.7(94)	6.4±0.17(149)	22.4±2.9(89)
KY1061	*BAT1 bat2::URA3 LEU2* (multi-copy *ATF1*)	82.2±4.7(48)	17.7±2.1(26)	30.2±3.9(94)	20.1±0.3(467)	112.4±4.0(444)

[a]Data are mean ±SD of three independent experiments

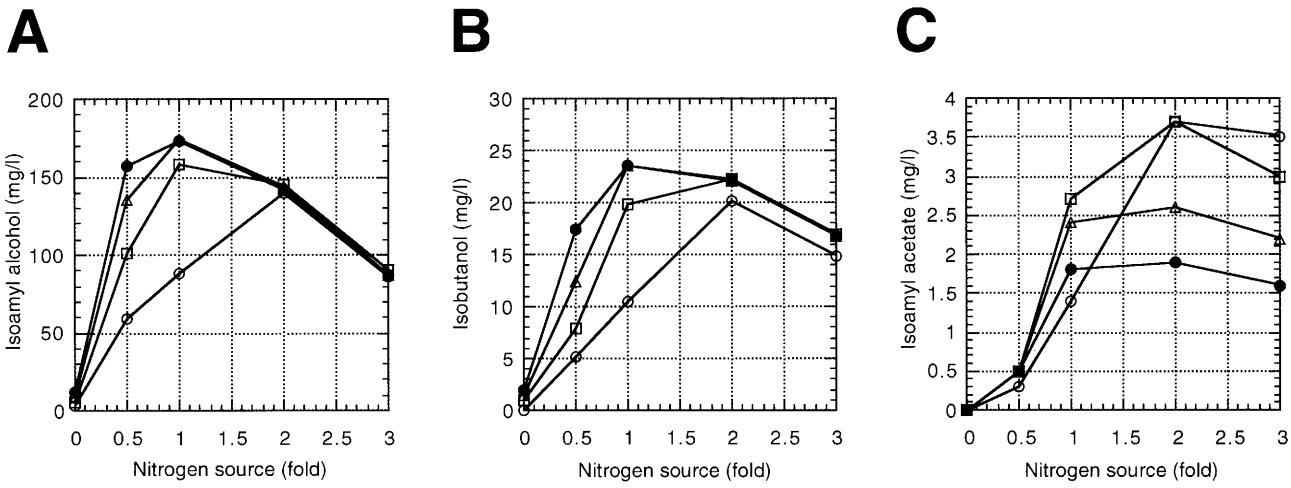

Fig. 1 Effect of nitrogen source on **A** isoamyl alcohol, **B** isobutanol, and **C** isoamyl acetate production. YHY554 strain was grown in the synthetic medium containing various concentrations of nitrogen source for 24 h (*open circles*), 48 h (*open squares*), 72 h (*open triangles*), or 96 h (*solid circles*)

not shown), since this mutant still exhibits residual activity of *Bat1p* (Kispal et al. 1996). Compared to the control strain KY1059, the *bat2* null mutant KY1058 exhibited a 40% and 72% decrease in the production of isoamyl alcohol and isobutanol, respectively, and a 58% fall in isoamyl acetate yield. These results are consistent with the notion that production of isoamyl acetate is dependent on the concentration of isoamyl alcohol (Calderbank and Hammond 1994). Overproduction of *BAT2* (KY1060 strain) resulted in 1.3-fold and 2.2-fold increase in production of isoamyl alcohol and isobutanol, respectively, and a 1.5-fold increase in isoamyl acetate yield. The yields of *n*-propanol and ethyl acetate were not changed by either the disruption or overexpression of the *BAT2* gene. These data showed that the *BAT2* gene product plays a significant role in branched-chain alcohols and isoamyl acetate production.

Modified expression of *BAT2* and *ATF1* genes alters the ratio of branched-chain alcohols/isoamyl acetate

Since the *bat2* null mutant KY1058 had no effect of growth rate, we prepared a new strain that did not have a flavor profile similar to previous mutants, using modified expression of *BAT2* and *ATF1* genes. To alter the production ratio between branched-chain alcohols and isoamyl acetate without a change in growth rate, we constructed transformant KY1061 of the *bat2* null mutant that overexpressed the *ATF1* gene. This strain was cultured at 30°C for 4 days in YPD10 medium containing 0.3 mg/ml G418 followed by analysis as above. Similar to the results of the *bat2* null mutant KY1058, no growth delay of these transformants was detected (data not shown). Transformant KY1061 exhibited a decrease in the production of isoamyl alcohol and isobutanol of 52% and 74%, respectively, and 4.7- and 4.4-fold increases in isoamyl acetate and ethyl acetate yields, respectively (Table 3). These data demonstrate that transformant KY1061 of the *bat2* null mutant with overexpression of the *ATF1* gene produces a low amount of branched-chain alcohols and high amount of isoamyl acetate without changes in growth, suggesting that modified expression of *BAT2* and *ATF1* genes can significantly alter both the proportion of different branched-chain alcohols and production of isoamyl acetate.

Effect of nitrogen source on production of branched-chain alcohols and isoamyl acetate

To examine the effect of nitrogen source on the production of branched-chain alcohols and isoamyl acetate, we performed fermentation tests using synthetic medium containing various concentrations (0, 0.5, 1, 2, and 3-fold) of nitrogen sources (amino acids and citrate ammonium). Strain YHY554 was cultured at 30°C under anaerobic conditions and the culture samples were collected every 24 h. The branched-chain alcohols and isoamyl acetate concentrations in these culture supernatants were determined (Fig. 1); at 96 h, the concentration of both isoamyl alcohol and isobutanol increased in the presence of high concentrations of nitrogen source until the concentration of nitrogen source reached 1-fold (Fig. 1A, B). Once this point was reached, further addition of nitrogen source reduced the yields of both isoamyl alcohol and isobutanol. The profiles of isoamyl alcohol and isobutanol production were almost identical.

As shown in Fig. 1C, the concentration of isoamyl acetate reached a maximum at 48 h at all nitrogen source levels, and decreased thereafter. This reduction may be due to evaporation, or to hydrolysis due to esterase activity (Fukuda et al. 1998). Thus, the concentration of isoamyl acetate at its maximum level (i.e., at 48 h) was compared to that of isoamyl alcohol at 48 h. Isoamyl acetate production increased as the amount of nitrogen source increased until the concentration of nitrogen source reached 2-fold, and slightly diminished at 3-fold nitrogen source. In contrast, the concentration of isoamyl alcohol reached a maximum at 1-fold of nitrogen source. After this time, this concentration decreased (Fig. 1A). These data suggest that when different amounts of nitrogen source were used, the production profile of isoamyl acetate was different to that of isoamyl alcohol.

Transcriptional regulation by nitrogen source

A precursor of isoamyl alcohol, α-ketoisocaproate, is synthesized by both β-IPM dehydrogenase, encoded by the *LEU2* gene (Ratzkin and Carbon 1977), and branched-chain amino acid aminotransferase, encoded by the *BAT1* and *BAT2* genes (Eden et al. 1996; Kispal et al. 1996). This α-keto acid is converted to isovaleraldehyde by the decarboxylase encoded by *PDC1* and *KID1* genes for isoamyl alcohol production (Dickinson et al. 1997; Yoshimoto et al. 2001). The exact steps that determine the production of isoamyl alcohol, and regulation of gene expression by nitrogen source have not been investigated. To determine the mechanism of the influence of nitrogen source on isoamyl alcohol and isoamyl acetate production, we investigated the expression of leucine biosynthetic *LEU4* (Beltzer et al. 1986), *LEU1* (Hsu and Schimmel 1984), and *LEU2* genes, branched-chain amino acid aminotransferase (*BAT1* and *BAT2*) genes, decarboxylase (*PDC1* and *KID1*) genes, and alcohol acetyltransferase (*ATF1* and *ATF2*) genes (Fujii et al. 1994; Nagasawa et al. 1998). Total RNA was isolated from yeast cells grown to the same OD_{600} in exponential-phase and RNA blot analysis was performed. The transcriptional profiles of *LEU* genes and *BAT* genes changed in a manner similar to that of the production of isoamyl alcohol (Fig. 2). There was no clear relationship between expression of decarboxylase genes (*PDC1* and *KID1*) and isoamyl alcohol production because expression of these genes increased with increased concentrations of nitrogen source. Therefore, these data suggest that the change in isoamyl alcohol production by nitrogen source is dependent on transcriptional co-regulation between *LEU* genes and *BAT* genes.

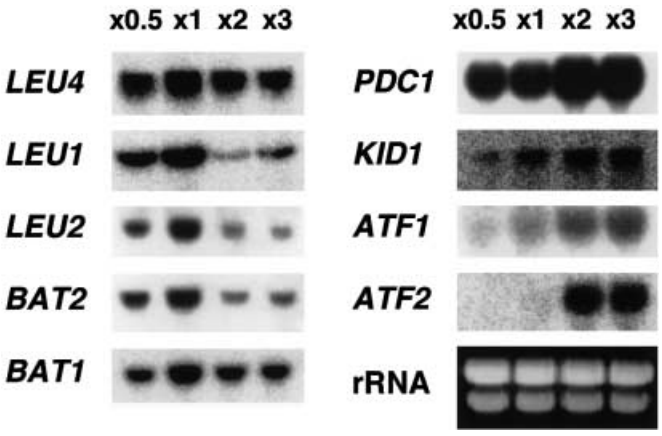

Fig. 2 RNA blot analysis of total RNA from samples grown at different ratios of nitrogen source using probes of leucine biosynthetic (*LEU4*, *LEU1*, and *LEU2*), branched-chain amino acid aminotransferase (*BAT1* and *BAT2*) genes, decarboxylase (*PDC1* and *KID1*) genes, and alcohol acetyltransferase (*ATF1* and *ATF2*) genes. Amounts of RNA loaded on the gel were compared by examining 18S and 25S ribosomal RNA levels

On the other hand, the synthesis of isoamyl acetate is closely related to that of isoamyl alcohol. Conditions associated with optimal production include both the level of isoamyl alcohol and transcription of *ATF* genes. RNA blot analysis indicated that *ATF1* and *ATF2* transcription increased with increased concentrations of nitrogen source (Fig. 2). Since isoamyl acetate production increased with increasing concentrations of nitrogen source until saturation of the system at 2-fold nitrogen source, these results suggest that isoamyl acetate production up to 1-fold nitrogen source depends on the production of its precursor isoamyl alcohol and *ATF* transcription while that from 1-fold to 2-fold nitrogen source depends on *ATF* transcription. Further addition of nitrogen source slightly decreased isoamyl acetate production. Since at this concentration of nitrogen source, *ATF* transcription is still activated by nitrogen source, the results suggest that the effect of isoamyl alcohol production at 3-fold nitrogen source is higher than that of *ATF* transcription. These data suggest that *ATF* transcription increases with increased concentrations of the nitrogen source and that isoamyl acetate production is dependent on both isoamyl alcohol production and *ATF* transcription.

Discussion

S. cerevisiae contains mitochondrial and cytosolic branched-chain amino acid aminotransferases encoded by the *BAT1* and *BAT2* genes, respectively (Eden et al. 1996; Kispal et al. 1996). In this study, we demonstrated that the *BAT2* gene product plays an important role in the production of branched-chain alcohols and isoamyl acetate and is involved in the Ehrlich pathway (Rainbow 1970). This pathway, an amino catabolic pathway, includes transamination, decarboxylation, and dehydrogenation processes, which result in the formation of branched-chain alcohols. *BAT* genes were cloned after cloning of *LEU* genes. Genes in the L-leucine biosynthetic pathway were cloned by complementing their mutants, which have an auxotrophy requirement for L-leucine. These mutants showed an auxotrophy requirement for L-leucine by single mutation. However, two genes exist for branched-chain amino acid aminotransferase. Thus, auxotrophy requirement for L-leucine was not detected when either of two genes was mutated. The null mutant, in which both genes are disrupted, showed auxotrophy requirement for L-leucine, which explains the delay in cloning *BAT* genes. Analysis of *BAT* genes should enhance our understanding of the production of branched-chain alcohols through the Ehrlich pathway.

To alter the synthesis of branched-chain alcohols and isoamyl acetate, we modified the expression of genes involved in their production. These studies could potentially lead to the development of a variety of yeast strains with diverse flavor profiles. To screen a yeast strain producing diverse profiles of branched-chain alcohols and isoamyl acetate, mutation of genes involved in the L-leucine biosynthetic pathway or mutations resistant to L-leucine analogues were used. Mutants of genes involved in the L-leucine biosynthetic pathway resulted in not only a low amount of isoamyl alcohol and isoamyl acetate, but also insufficient growth rate (data not shown). On the other hand, mutants resistant to L-leucine analogues have been shown to produce large amounts of isoamyl alcohol and isoamyl acetate (Ashida et al. 1987; Watanabe et al. 1990). Therefore, it is virtually impossible to screen mutants that result in low amounts of branched-chain alcohols and high levels of isoamyl acetate without changing the growth rate. However, we demonstrated that a transformant of a *bat2* null mutant overexpressing the *ATF1* gene produced low amounts of higher alcohols and high amounts of isoamyl acetate without changing the growth rate. We believe that this modification will enhance the diversity of flavors in the fermented products and lead to the development of new types of yeast-fermented alcoholic beverages.

Branched-chain alcohols are synthesized by decarboxylation and reduction of the corresponding α-keto acids. The latter are produced through transamination of branched-chain amino acids synthesis or by carbohydrate catabolism. In this pathway, formation of α-keto acids is regulated by three enzymes: β-IPM (encoded by *LEU2* gene), decarboxylase (encoded by *PDC1* and *KID1* genes) and branched-chain amino acid aminotransferase (encoded by *BAT* genes). We investigated the regulation of these three enzymes in isoamyl alcohol production by using various amounts of nitrogen source. Fermentation tests using different amounts of nitrogen source and RNA blot analyses demonstrated that the production of isoamyl alcohol correlated with the expression of both the *LEU* and *BAT* genes, but not that of *PDC1* and *KID1* genes, and that the expression of *LEU* and *BAT* genes is co-regulated by nitrogen source. These data suggested that isoamyl alcohol production influenced by nitrogen source depends on transcription of L-leucine biosynthetic and branched-chain amino acid aminotransferase-encoding genes.

General and specific mechanisms control the synthesis pathways of branched-chain amino acids (Hinnebusch 1990, 1992). Examples of the former control mechanism include repression and depression of common enzymes in different amino acid pathways. The latter control mechanism operates on specific amino acid pathways and involves end-products and intermediates of amino acid biosynthesis pathways. Amino acid biosynthesis is regulated at both transcriptional and translational levels. It has been reported that the *LEU1*, *LEU2* and *LEU4* genes require a positive regulator encoded by *LEU3*, and a Leu3p-dependent protein-DNA complex has been shown to form specifically with a G+C-rich palindromic decanucleotide sequence 5′-CCGG-NNCCGG-3′ present in the 5′-noncoding regions of these three genes (Friden and Schimmel 1988). These *LEU* genes also contain the recognition sequence 5′-TGACTC-3′ for the transcription activator Gcn4p, which mediates general amino acid control (Arndt and Fink 1986). Computer analysis of the promoters showed that the promoter of the *BAT2* gene does not contain a clear recognition sequence 5′-CCGGNNCCGG-3′ and 5′-TGACTC-3′. However, we detected a similar sequence 5′-TGACAC-3′ at position –323 to –318 and position –293 to –288, and 5′-CCGGAACCAT-3′ at position –366 to –357 of the promoter of *BAT2* gene. On the other hand, the promoter of the *BAT1* gene contains both recognition sequence 5′-TGACTC-3′ at position –171 to –166 and 5′-CCGGNNCCGG-3′ at position –147 to –138 of this promoter. Furthermore, as a result of DNA microarray analysis, Natarajan et al. (2001) reported that the *BAT1* and *BAT2* genes are Gcn4p targets. Although it is unclear if *BAT2* gene expression is regulated through these recognition sequences, genes in the leucine metabolic pathway, including *BAT* genes, could be co-regulated by Leu3p and/or Gcn4p. Promoter deletion analysis of *BAT* genes should further enhance our understanding of the transcriptional co-regulation of these genes.

In *S. cerevisiae*, the FGM (fermentable-growth-medium-induced) pathway plays an important role in the utilization of a fermentable carbon source and all other growth nutrients for activation. Activation of the FGM pathway is not mediated by cAMP-dependent protein kinase. The *SCH9* gene, encoding a protein kinase that shows homology to the catalytic subunits of cAMP-dependent protein kinase, is thought to be the target of the

FGM pathway and is required for nitrogen-induced activation of the FGM pathway (Crauwels et al. 1997). Analysis of *ATF1* expression in a *sch9* mutant suggested that Sch9p is involved in *ATF1* transcriptional activation (Fujiwara et al. 1999). Although it is demonstrated that *ATF1* expression is mediated by Sch9p in the FGM pathway, the relationship between *ATF1* expression and nitrogen source has not yet been verified. Our results indicate that *ATF1* expression is activated by nitrogen source, suggesting that *ATF1* expression is activated by nitrogen source through Sch9p in the FGM pathway.

In conclusion, our study has clearly demonstrated the important role of the *BAT2* gene product, cytosolic branched-chain amino acid aminotransferase, for the production of branched-chain alcohols and isoamyl acetate, the alteration of such production by modified expression of *BAT2* and *ATF1* genes, the effect of nitrogen source on production of branched-chain alcohols and isoamyl acetate, and transcriptional regulation by nitrogen source of genes involved in the synthesis of isoamyl alcohol and isoamyl acetate. Our results point to the enormous effect that gene technology is likely to have on our understanding of the Ehrlich pathway, paving the way for the production of high quality flavor. Further studies of the single mutant strain of *BAT1* gene and double mutation of *BAT1* and *BAT2* genes should advance our understanding of branched-chain alcohols and isoamyl acetate formation, and of the Ehrlich pathway.

Acknowledgement We thank Chiori Ito for technical assistance.

References

Amberg DC, Botstein D, Beasley EM (1995) Precise gene disruption in *Saccharomyces cerevisiae* by double fusion polymerase chain reaction. Yeast 11:1275–1280

Arndt K, Fink GR (1986) GCN4 protein, a positive transcription factor in yeast, binds general control promoters at all 5′ TGACTC 3′ sequences. Proc Natl Acad Sci USA 83: 8516–8520

Ashida S, Ichikawa E, Suginami K, Imayasu S (1987) Isolation and application of mutants producing sufficient isoamyl acetate, a sake flavor component. Agric Biol Chem 51:2061–2065

Beltzer JP, Chang LF, Hinkkanen AE, Kohlhaw GB (1986) Structure of yeast *LEU4*. The 5′ flanking region contains features that predict two modes of control and two productive translation starts. J Biol Chem 261:5160–5167

Botstein D, Falco SC, Stewart SE, Brennan M, Scherer S, Stinchcomb DT, Struhl K, Davis RW (1979) Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene 8:17–24

Broach JR, Strathern JN, Hicks JB (1979) Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene 8:121–133

Calderbank J, Hammond JRM (1994) Influence of higher alcohol availability on ester formation by yeast. J Am Soc Brew Chem 52:84–90

Crauwels M, Donaton MC, Pernambuco MB, Winderickx J, de Winde JH, Thevelein JM (1997) The Sch9 protein kinase in the yeast *Saccharomyces cerevisiae* controls cAPK activity and is required for nitrogen activation of the fermentable-growth-medium-induced (FGM) pathway. Microbiology 143: 2627–2637

Dickinson JR, Norte V (1993) A study of branched-chain amino acid aminotransferase and isolation of mutations affecting the catabolism of branched-chain amino acids in *Saccharomyces cerevisiae*. FEBS Lett 326:29–32

Dickinson JR, Lanterman MM, Danner DJ, Pearson BM, Sanz P, Harrison SJ, Hewlins MJE (1997) A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J Biol Chem 272: 26871–26878

Eden A, Simchen G, Benvenisty N (1996) Two yeast homologs of *ECA39*, a target for c-Myc regulation, code for cytosolic and mitochondrial branched-chain amino acid aminotransferases. J Biol Chem 271:20242–20245

Eden A, Van Nedervelde L, Drukker M, Benvenisty N, Debourg A (2001) Involvement of branched-chain amino acid aminotransferases in the production of fusel alcohols during fermentation in yeast. Appl Microbiol Biotechnol 55:296–300

Elion EA, Warner JR (1984) The major promoter element of rRNA transcription in yeast lies 2 kb upstream. Cell 39:663–673

Friden P, Schimmel P (1988) *LEU3* of *Saccharomyces cerevisiae* activates multiple genes for branched-chain amino acid biosynthesis by binding to a common decanucleotide core sequence. Mol Cell Biol 8:690–697

Fujii T, Nagasawa N, Iwamatsu A, Bogaki T, Tamai Y Hamachi M (1994) Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. Appl Environ Microbiol 60:2786–2792

Fujii T, Kobayashi O, Yoshimoto H, Furukawa, S Tamai Y (1997) Effect of aeration and unsaturated fatty acids on expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase gene. Appl Environ Microbiol 63:910–915

Fujiwara D, Yoshimoto H, Sone H, Harashima S, Tamai Y (1998) Transcriptional co-regulation of *Saccharomyces cerevisiae* alcohol acetyltransferase gene, *ATF1* and Δ-9 fatty acid desaturase gene, *OLE1* by unsaturated fatty acids. Yeast 14:711–721

Fujiwara D, Kobayashi O, Yoshimoto H, Harashima S, Tamai Y (1999) Molecular mechanism of the multiple regulation of the *Saccharomyces cerevisiae ATF1* gene encoding alcohol acetyltransferase. Yeast 15:1183–1197

Fukuda K, Yamamoto N, Kiyokawa Y, Yanagiuchi T, Wakai Y, Kitamoto K, Inoue Y, Kimura A (1998) Brewing properties of sake yeast whose *EST2* gene encoding isoamyl acetate-hydrolyzing esterase was disrupted. J Ferment Bioeng 85:101–106

Hammond JRM (1993) Fusel alcohols. In: Rose AH, Harrison JS (eds) The yeasts. Academic Press, London, pp 34–36

Hinnebusch AG (1990) Transcriptional and translational regulation of gene expression in the general control of amino-acid biosynthesis in *Saccharomyces cerevisiae*. Prog Nucleic Acid Res Mol Biol 38:195–240

Hinnebusch AG (1992) General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*. In: Jones EW, Pringle JR, Broach JR (eds) The molecular and cellular biology of the yeast *Saccharomyces*, vol 2. Gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp 387–392

Hirata D, Hiroi T (1991) Genes that cause overproduction of isoamyl alcohol by increased gene-dosage effect in *Saccharomyces cerevisiae*. Agric Biol Chem 55:919–924

Hsu YP, Schimmel P (1984) Yeast *LEU1*: repression of mRNA levels by leucine and relationship of 5′-noncoding region to that of *LEU2*. J Biol Chem 259:3714–3719

Ichihara A, Koyama E (1966) Transaminase of branched chain amino acids. J Biochem 59:160–169

Iersel MFM, Eppink MHM, Berkel WJH, Rombouts FM, Abee T (1997) Purification and characterization of a novel NADP-dependent branched-chain alcohol dehydrogenase from *Saccharomyces cerevisiae*. Appl Environ Microbiol 63:4079–4082

Kispal G, Steiner H, Court DA, Rolinski B, Lill R (1996) Mitochondrial and cytosolic branched-chain amino acid transaminases from yeast, homologs of the myc oncogene-regulated Eca39 protein. J Biol Chem 271:24458–24464

Nagasawa N, Bogaki T, Iwamatsu A, Hamachi M, Kumagai C (1998) Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (*ATF2*) from *Saccharomyces cerevisiae* Kyokai No.7. Biosci Biotech Biochem 62:1852–1857

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, Marton MJ (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol Cell Biol 21:4347–4368

Oshita K, Kubota M, Uchida M, Ono M (1995) Clarification of the relationship between fusel alcohol formation and amino acid assimilation by brewing yeast using ^{13}C-labeled amino acid. Proceedings of the 25th European Brewery Convention Congress, pp 387–394

Rainbow C (1970) Brewer's yeasts. In: Harrison JS, Rose AH (eds) The yeasts vol 3, Academic Press, New York, pp 190–199

Ratzkin B, Carbon J (1977) Functional expression of cloned yeast DNA in *Escherichia coli*. Proc Natl Acad Sci USA 74:487–491

Singh R, Kunkee RE (1976) Alcohol dehydrogenase activities of wine yeasts in relation to higher alcohol formation. Appl Environ Microbiol 32:666–670

Tamai Y (1996) Alcohol acetyl transferase genes and ester formation in brewer's yeast. In: Takeoka GR, Teranishi R, Williams PJ, Kobayashi A (eds) Biotechnology for improved foods and flavours. American Chemical Society, Washington D.C. pp 196–205

Wach A (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast 12:259–265

Watanabe M, Fukuda K, Asano K, Ohta S (1990) Mutants of bakers' yeasts producing a large amount of isobutyl alcohol or isoamyl alcohol, flavour components of bread. Appl Microbiol Biotech 34:154–159

Yamashita I, Fukui S (1985) Transcriptional control of the sporulation-specific glucoamylase gene in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol 5:3069–3073

Yoshimoto H, Fujiwara D, Momma T, Sone H, Kaneko Y, Tamai Y (1998) Characterization of the *ATF1* and Lg-*ATF1* genes encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus*. J Ferment Bioeng 86:15–20

Yoshimoto H, Fujiwara D, Momma T, Tanaka K, Sone H, Nagasawa N, Tamai Y (1999) Isolation and characterization of the *ATF2* gene encoding alcohol acetyltransferase II in the bottom fermenting yeast *Saccharomyces pastorianus*. Yeast 15:409–417

Yoshimoto H, Fukushige T, Yonezawa T, Sakai Y, Okawa K, Iwamatsu A, Sone H, Tamai Y (2001) Pyruvate decarboxylase encoded by *PDC1* gene contributes, at least partially, to the decarboxylation of α-ketoisocaproate for isoamyl alcohol formation in *Saccharomyces cerevisiae*. J Biosci Bioeng 92:83–85