Applied Microbiology and Biotechnology (2020) 104:7915–7925
https://doi.org/10.1007/s00253-020-10800-y

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Effect of the Ala234Asp replacement in mitochondrial branched-chain amino acid aminotransferase on the production of BCAAs and fusel alcohols in yeast

Jirasin Koonthongkaew[1] · Yoichi Toyokawa[1] · Masataka Ohashi[2] · Christopher R. L. Large[3] · Maitreya J. Dunham[3] · Hiroshi Takagi[1]

Received: 27 April 2020 / Revised: 17 July 2020 / Accepted: 27 July 2020 / Published online: 10 August 2020

Abstract

In the yeast *Saccharomyces cerevisiae*, the mitochondrial branched-chain amino acid (BCAA) aminotransferase Bat1 plays an important role in the synthesis of BCAAs (valine, leucine, and isoleucine). Our upcoming study (Large et al. bioRχiv. 10.1101/2020.06.26.166157, Large et al. 2020) will show that the heterozygous tetraploid beer yeast strain, Wyeast 1056, which natively has a variant causing one amino acid substitution of Ala234Asp in Bat1 on one of the four chromosomes, produced higher levels of BCAA-derived fusel alcohols in the brewer's wort medium than a derived strain lacking this mutation. Here, we investigated the physiological role of the A234D variant Bat1 in *S. cerevisiae*. Both *bat1*Δ and *bat1*^{A234D} cells exhibited the same phenotypes relative to the wild-type Bat1 strain—namely, a repressive growth rate in the logarithmic phase; decreases in intracellular valine and leucine content in the logarithmic and stationary growth phases, respectively; an increase in fusel alcohol content in culture medium; and a decrease in the carbon dioxide productivity. These results indicate that amino acid change from Ala to Asp at position 234 led to a functional impairment of Bat1, although homology modeling suggests that Asp234 in the variant Bat1 did not inhibit enzymatic activity directly.

Key points

- *Yeast cells expressing Bat1*^{A234D} *exhibited a slower growth phenotype.*
- *The Val and Leu levels were decreased in yeast cells expressing Bat1*^{A234D}.
- *The A234D substitution causes a loss-of-function in Bat1.*
- *The A234D substitution in Bat1 increased fusel alcohol production in yeast cells.*

Keywords Yeast · *Saccharomyces cerevisiae* · Mitochondrial branched-chain amino acid aminotransferase Bat1 · Branched-chain amino acids · Fusel alcohols · Beer brewing

Introduction

Branched-chain amino acids (BCAAs; valine, leucine, and isoleucine) are essential and functional in humans (Mori et al. 1988; Hiroshige et al. 2001; Calder 2006; Howatson et al. 2012), leading to human diseases (Yamada et al. 2014; Wang et al. 2015; Manoli and Venditti 2016) or abnormal phenotypes in mice (She et al. 2007; She et al. 2010) when genes involved in BCAA catabolism are disrupted. As well, several million tons of BCAAs are produced every year using microorganisms (Ikeda 2003) and are utilized in a wide range of industries (Sahm et al. 1995; Park et al. 2007). Hence, it is important to further clarify the metabolic regulation of BCAAs to help elucidate their physiological significance

Jirasin Koonthongkaew and Yoichi Toyokawa contributed equally to this work.

✉ Hiroshi Takagi
hiro@bs.naist.jp

[1] Division of Biological Science, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma, Nara 630-0192, Japan

[2] Nara Prefecture Institute of Industrial Development, 129-1 Kashiwagi-cho, Nara, Nara 630-8031, Japan

[3] Department of Genome Sciences, University of Washington, 3720 15th Ave NE, Seattle, WA 98195, USA

and find ways to increase their industrial production. Although mammalian cells cannot synthesize BCAAs by themselves, the yeast *Saccharomyces cerevisiae*, which is widely used as a model for higher eukaryotes, has the ability to produce BCAAs (Fig. 1). Inside the yeast mitochondria, both valine (Val) and leucine (Leu) are primarily biosynthesized from two pyruvates while isoleucine (Ile) is produced from an initial α-ketobutyrate molecule. Pyruvate and α-ketobutyrate are then converted into α-ketoisovalerate (KIV) and α-keto-β-methylvalerate (KMV), respectively,

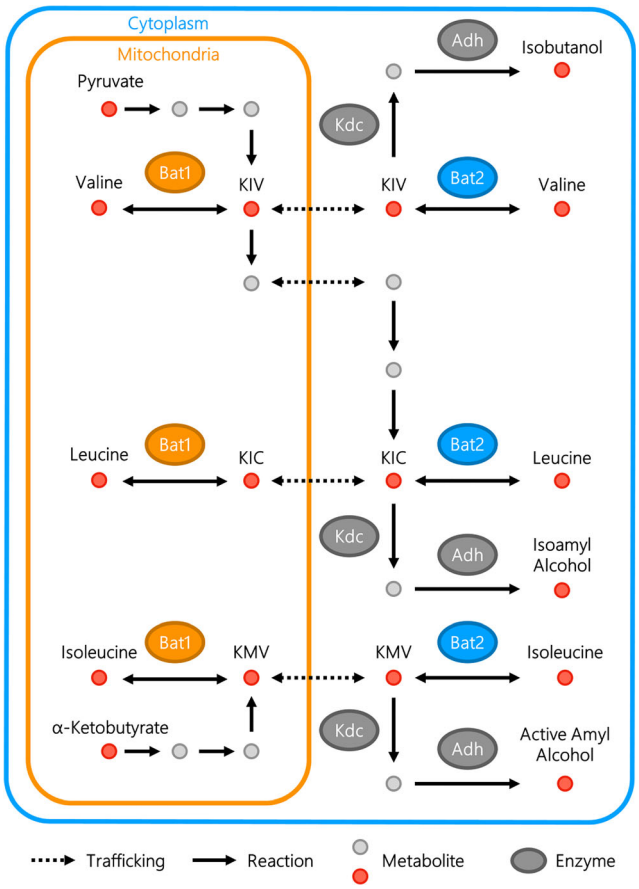

Fig. 1 Schematic metabolism of BCAAs in *S. cerevisiae*. Metabolism of BCAAs in *S. cerevisiae* is summarized schematically. Pyruvate and α-ketobutyrate are individually converted into α-ketoisovalerate (KIV) and α-keto-β-methylvalerate (KMV), respectively, by the reactions of identical enzymes in the mitochondria. KIV is further converted to α-ketoisocaproate (KIC) mainly in cytosol. KIV, KIM, and KIC are then transaminated to valine (Val), isoleucine (Ile), and leucine (Leu), respectively, by branched-chain amino acid aminotransferases (BCATs) localized in the mitochondria (Bat1) and cytoplasm (Bat2). The BCATs also catabolize BCAAs to α-keto acids (KIC, KMV, and KIC) by the reaction of oxidative deamination in the cytoplasm or mitochondria. KIV, KMV, and KIC are further converted into isobutanol, active amyl alcohol, and isoamyl alcohol, respectively, by the reaction of α-keto acid decarboxylase (KDC) and alcohol dehydrogenase (AHD). The double arrows indicated bidirectional reaction which is catalyzed by one enzyme, whereas arrows indicated one-direction reaction by each enzyme. The dash lines indicate transport between the mitochondria and cytoplasm by the known or unidentified transporter protein(s)

through the same pathway. In contrast, α-ketoisocaproate (KIC) is synthesized from KIV. KIV, KMV, and KIC are finally transaminated to Val, Ile, and Leu, respectively, by the mitochondrial and cytoplasmic BCAA aminotransferases (BCATs) Bat1 and Bat2, respectively.

Fusel alcohols, which are known as important flavor components in beer (Pires et al. 2014), are generated from BCAAs via the Ehrlich pathway during the fermentation process in yeast (Hazelwood et al. 2008). In the Ehrlich pathway, Val, Leu, and Ile are first transaminated to KIV, KIC, and KMV, respectively, by Bat1 or Bat2. These α-keto acids (KIV, KIC, and KMV) are decarboxylated by α-ketoacid decarboxylases (KDCs) and continuously reduced by NAD(P)H-dependent alcohol dehydrogenases (ADHs) converting them in the cytoplasm to isobutanol, isoamyl alcohol (3-methylbutan-1-ol), and active amyl alcohol (2-methylbutan-1-ol), respectively. Such fusel alcohols are applied to various industrial fields. Notably, isobutanol is of interest as a next-generation biofuel alternative to bioethanol due to its higher combustion power, lower aqueous miscibility, and a weaker corrosivity compared with ethanol, the first-generation biofuel that is currently in the widest use (Weber et al. 2010). Since the productivity of isobutanol is low in *S. cerevisiae*, several attempts have been made to obtain yeast strains with higher isobutanol productivity by genetic engineering (Lilly et al. 2006; Avalos et al. 2013; Hammer and Avalos 2017; Park and Hahn 2019; Wess et al. 2019). Particular attention has also been paid to isoamyl alcohol as a biofuel for use in homogeneous charge compression ignition (HCCI) engines (Yang et al. 2010; Tsujimura et al. 2011; Welz et al. 2012). Isoamyl alcohol has also garnered interest because it is a precursor of isoamyl acetate, which is widely used in food industries for its banana flavor characteristics (Romero et al. 2005). In order to improve the productivity of isoamyl acetate in yeast fermentation broth, some studies have examined the use of metabolic engineering approaches (Ashida et al. 1987; Hirata et al. 1992). Therefore, fusel alcohol production is important for the development of the biofuel industry, in addition to being of general interest to improve our understanding of the metabolic mechanisms operational in *S. cerevisiae*.

Bat1 and Bat2, which are the key enzymes in the biosynthesis of BCAAs, catalyze the reaction of a bidirectional transamination between α-keto acids (KIV, KMV, and KIC) and BCAAs. In addition, the *BAT1* gene is highly expressed during the logarithmic growth phase and repressed during the stationary growth phase, whereas the *BAT2* gene is highly expressed in the stationary phase and repressed during the logarithmic growth (Eden et al. 1996). Though Bat1 and Bat2 share homology with each other (77% amino acid identity), the *BAT1* gene is highly expressed under the biosynthetic conditions, such as in medium containing ammonium as a nitrogen source, while the *BAT2* gene is induced under the catabolic conditions,

such as in the presence of BCAAs (Colón et al. 2011). Moreover, the expression of the *BAT1/2* genes and the bidirectional catalytic activities of BCATs are controlled in a complex fashion by yeast in response to the different kinds of amino acids, the nutritional status of the yeast cell, and the effects of several transcriptional activators (González et al. 2017). However, most of the studies involving the *BAT1* and *BAT2* genes have used knockout mutant strains with few attempting to investigate the effect of variant BCATs on cellular phenotypes. Therefore, in order to understand the control system of BCATs in *S. cerevisiae*, it is necessary to clarify the relationship between the primary structure and physiological functions of BCATs.

Recently, Large et al. (2020) found that isobutanol and amyl alcohol contents were increased in fermentation broth prepared with a tetraploid beer yeast strain, Wyeast 1056, which natively has one amino acid change from Ala to Asp at position 234 in Bat1, compared with the strain without this mutation. In addition, the Wyeast 1056 strain showed an increase in isobutanol and amyl alcohol production levels and a decrease in diacetyl and 2,3-pentanedione production in the fermentation broth, compared with the strain without this mutation (Large et al. 2020). These findings strongly suggest that the A234D substitution in Bat1 affects the physiological function of Bat1, particularly with regard to BCAA biosynthesis and fusel alcohol production. Hence, in this study, we aimed to clarify the physiological significance of the A234D substitution in Bat1 in the industrial beer yeast strain.

Materials and methods

Strains and culture media

A *S. cerevisiae* BY4741*bat1*Δ strain (BY4741*bat1::kanMX6*) was used as a host strain for the construction of various transformants. An *Escherichia coli* DH5α strain (*F*-, *φ80dlacZΔM15*, Δ(*lacZYA-argF*)*U169*, *deoR*, *recA1*, *endA1*, *hdR17* (r_k^-, m_k^+), *phoA*, *supE44*, λ^-, *thi-1*, *gyrA96*, *relA1*) was used as a host for the construction and extraction of plasmids. Unless otherwise noted, yeast strains were cultured in a nutrient-rich YPD medium {10 g yeast extract [Becton, Dickinson, and Company (BD), Franklin Lakes, NJ], 20 g peptone (BD), and 20 g glucose (Nacalai Tesque, Kyoto, Japan) per liter} or a synthetic dextrose (SD) minimal medium [1.7 g yeast nitrogen base without amino acid (BD), 5 g ammonium sulfate (Nacalai Tesque), and 20 g glucose at pH 6.0 per liter]. An *E. coli* DH5α strain was cultivated in LB medium [5 g yeast extract, 10 g tryptone (BD), and 10 g NaCl (Nacalai Tesque) per liter].

Construction of plasmid and transformants

The Bat1 protein was expressed in *S. cerevisiae* BY4741*bat1*Δ (BY4741 *bat1*Δ*::kanMX6*), which was constructed from a haploid *S. cerevisiae* strain BY4741 (*MATa his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) used in the previous study (Takpho et al. 2018). Plasmid pRS416-*BAT1* fused with GFP was used as the Bat1-expressing plasmid; the construction of which was based on pRS416 (*CEN6*, *URA3*) (Takpho et al. 2018). The $Bat1^{A234D}$-expressing plasmid (pRS-$BAT1^{A234D}$) was constructed with PCR-mediated site-directed mutagenesis by using pRS416-*BAT1* as a template. To introduce the mutation in the *BAT1* gene, PCR was performed with the following primers: *BAT1*_QC_A234D_F (5′-TAC CTC AAC TAC AAG ATG CCA AAA GAG GGT A-3′) and *BAT1*_QC_A234D_R (5′-TAC CCT CTT TTG GCA TCT TGT AGT TGA GGT A-3′). Double-underlined nucleotides indicate the positions inducing amino acid substitution from Ala234 to Asp234 in Bat1. The resultant PCR products were treated with *Dpn*I and subsequently introduced into *E.coli* DH5α cells (Inoue et al. 1990). DNA sequences of the PCR products and plasmid constructs were verified by using a DNA sequencer (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems, Waltham, MA). In order to complement the auxotrophic phenotype of BY4741*bat1*Δ strains for expressing the Bat1 variants in the cell, pRS416-based plasmids were co-transformed with plasmid pRS415-Cg*HIS3MET15*, which was constructed based on pRS415(*CEN6*, *LEU2*) used in the previous study (Takpho et al. 2018). Transformation of yeast cells was carried out based on a high-efficiency transformation method (Burke et al. 2000). The transformants were selected on SD agar plates, and transformed colonies were confirmed by PCR analysis (for plasmids) prior to use in subsequent experiments.

Spot test analysis

Yeast cells were precultured in 5 mL of SD medium at 30 °C for 18 h. The cell suspensions were collected and diluted to an optical density 600 nm (OD_{600}) of 5 per mL and resuspended in distilled water. Each of the cell suspensions was further diluted 10-fold serially and 2 μL of each diluted suspension was dropped on the SD medium containing glucose 2, 10, 20, and 30%.

Measurement of cell growth

Yeast cells were pre-incubated in 5 mL of SD medium at 30 °C for 16 h. The cell suspensions were then transferred to 50 mL of SD medium with an initial OD_{600} of 0.1 per mL. The OD_{600} was measured every 4 h until the culture reached the stationary growth phase.

Fermentation test

For measurement of fermentation rates, yeast cells were pre-incubated in 5 mL of SD medium at 30 °C for 16 h. The culture broth was transferred to 5 mL of SD liquid medium containing glucose (2 or 20%) and incubated at 18 °C under static conditions, which mimics the fermentative conditions in beer brewing. Fermentation progression was continuously monitored by measuring the weight of test tubes every 24 h for 14 days (the loss of media weight was determined as CO_2 evolution) (Bely et al. 1990).

Western blot analysis of the Bat1 protein

Protein extraction of yeast cells was performed using the alkaline extraction method (Zhang et al. 2011). Briefly, yeast cells were pre-incubated in 5 mL of SD medium at 30 °C for 16 h. The cell suspensions were harvested (OD_{600} = 1.0 per mL), dissolved in 0.8 mL of 2 M lithium-acetate, and continuously treated with 0.8 mL of 0.4 M NaOH. After keeping on ice for 5 min, the resultant precipitate was dissolved in 50 μL of HU buffer [0.2 M Tris-HCl (pH 6.8), 8 M urea, 5% SDS, 1 mM EDTA, 0.1 M DTT, 0.005% bromophenol blue] for preparing SDS-polyacrylamide gel electrophoresis (PAGE) samples. The Bat1 variant and the loading control Pgk1 proteins were detected with an anti-GFP mouse antibody (F. Hoffmann-La Roche, Basel, Switzerland) and an anti-Pgk1 mouse antibody (Thermo Fisher Scientific, Waltham, MA) at dilution rates of 1:5,000 and 1:20,000, respectively.

Measurement of intracellular BCAA contents

Yeast cells were pre-cultivated in 5 mL of SD liquid medium at 30 °C for 18 h. The cell cultures were recovered and transferred to 50 mL of SD medium with the initial OD_{600} of 0.1 per mL. After a 16- and 32-h incubation, the yeast cells were collected and resuspended in distilled water. The cell suspensions, adjusted to an OD_{600} of 10 per mL, were boiled at 100 °C for 20 min to extract intracellular amino acids. Cell debris were removed by centrifugation and the supernatant was filtrated by using a 0.2-μm syringe filter (Merck KGaA, Darmstadt, Germany). The filtrated extract was subjected to a LC/MS amino acid system (UF-Amino Station, Shimadzu, Kyoto, Japan) to quantify amino acid contents. Experimental procedures for analyzing amino acid content by LC/MS were conducted as reported previously (Shimbo et al. 2009). The content of each amino acid was expressed as a percentage of dry cell weight.

Measurement of fusel alcohol contents

Yeast cells were cultured in SD liquid medium using a 200-mL Erlenmeyer flask with shaking for 3 days at 30 °C, and fusel alcohol content was quantified by GC-MS. GC-MS analysis was performed on a GCMS-QP2010 SE (Shimadzu) equipped with an AOC-5000 PLUS autosampler. Helium was used as the carrier gas at a flow rate 1.28 mL per min. The initial temperature of the column was kept at 30 °C for 7 min, increased to 250 °C at a rate of 20 °C per min, and maintained at 250 °C for 5 min. The injector temperature was 200 °C, and injection was performed in the split mode (split ratio, 1:5). We used a standard solution comprising three kinds of pentyl alcohols (isobutanol, isoamyl alcohol, and active amyl alcohol) and n-amyl alcohol solution (500 μg per mL) as the internal standard solution. Two-hundred μL of the internal standard solution were added in distilled water to 1.8 mL of each standard solution (1–100 μg per mL) in distilled water in a 20-mL vial sealed with a PTFE septum cap. The supernatant from the yeast cultures was diluted appropriately in distilled water (hereafter called the sample solution) and added into a 20-mL vial with the internal standard using the same method as the standard solution. The vials of the standards and samples were treated at 70 °C for 30 min, and 2 mL of the gas phase in the vial was injected. The flavor components of the gas phase were separated on a DB-5MS column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Agilent Technologies) and determined by MS under selected ion monitoring (SIM) mode and quantified by the internal standard method. The mass spectrometer conditions were as follows: ionization energy, 70 eV; ion source temperature, 200 °C; interface temperature, 250 °C; SIM selected ions monitoring: isobutanol (m/z: 31, 74), isoamyl alcohol (m/z: 55, 70), active amyl alcohol (m/z: 57, 70), and n-amyl alcohol (m/z: 55, 70).

Homology modeling

The wild-type and the A234D variant Bat1 structures were constructed by homology modeling using the SWISS-MODEL (http://swissmodel.expasy.org/). The human BCAA aminotransferase, mBCAT (PBD ID: 1KTA), was used as a template. The amino acid sequence identity of the human mBCAT with the wild-type Bat1 was 43.77%. The structure of model A was used for the Ala234Asp variants of Bat1.

Statistical analysis

The values were the means and standard deviations of the results from the three independent experiments. The statistical analysis of significant difference was performed by non-repeated analysis of variance (ANOVA) followed by Bonferroni correction.

Results

Effects of the A234D substitution in Bat1 on the growth phenotype and BCAA production

The work in a companion study (Large et al. 2020) strongly suggested that an amino acid substitution in Bat1 from Ala to Asp at position 234 affected the fermentation ability of a beer yeast strain. To examine the effect of the amino acid substitution in Bat1, we first modified a laboratory strain (BY4741 bearing a deletion in *BAT1*) by transforming it with a plasmid expressing either the wild-type or modified variant of Bat1. Bat1 expression in the transformants was confirmed using a western blot for a GFP tag that was fused to the C-terminus of the Bat1 variant [BY4741 (Bat1-GFP, Bat1^{A234D}-GFP)], based on plasmid pRS416-*BAT1* fused to GFP constructed in our previous study (Takpho et al. 2018). Western blot analysis revealed that the wild-type and variant Bat1 were expressed in *S. cerevisiae* cells (Fig. 2a).

Next, to examine the effect of this variation in the *BAT1* gene on growth, we cultivated the BY4741*bat1*Δ strains bearing either one of the *BAT1* alleles [BY4741 (Bat1, Bat1^{A234D})] or an empty vector [BY4741*bat1*Δ (EV)] in SD medium and compared their cellular growth by monitoring the OD_{600} values of the cultures over time (Fig. 2b). BY4741*bat1*Δ (Bat1^{A234D}) and BY4741*bat1*Δ (EV) exhibited a slower growth phenotype than that of BY4741 (Bat1) expressing the wild-type Bat1, whereas all of the strains reached a comparable OD_{600} value after 32 h of growth. To further analyze the function of the Bat1 variant, we determined the intracellular BCAA contents of the yeast strains. It has been previously reported that Bat1 is highly expressed during the logarithmic growth phase and repressed during the stationary phase while Bat2 is only expressed during the stationary growth phase (Kispal et al. 1996; Eden et al. 1996). Therefore, yeast cells were collected at the logarithmic (16 h) and the stationary phase (32 h) of growth. As shown in Fig. 2c, the intracellular Val contents in BY4741*bat1*Δ (EV) and BY4741 (Bat1^{A234D}) were lower than that of BY4741 (Bat1) at the logarithmic phase. However, the amounts of Leu and Ile were similar between all of the strains. In contrast, the intracellular levels of Val and Leu were considerably decreased in BY4741*bat1*Δ (EV) and BY4741 (Bat1^{A234D}) when compared with BY4741 (Bat1) at the stationary phase. Similar to the previous experiment, there was no significant difference in the Ile content among the strains (Fig. 2d). These results suggest that the A234D substitution causes a loss-of-function in Bat1.

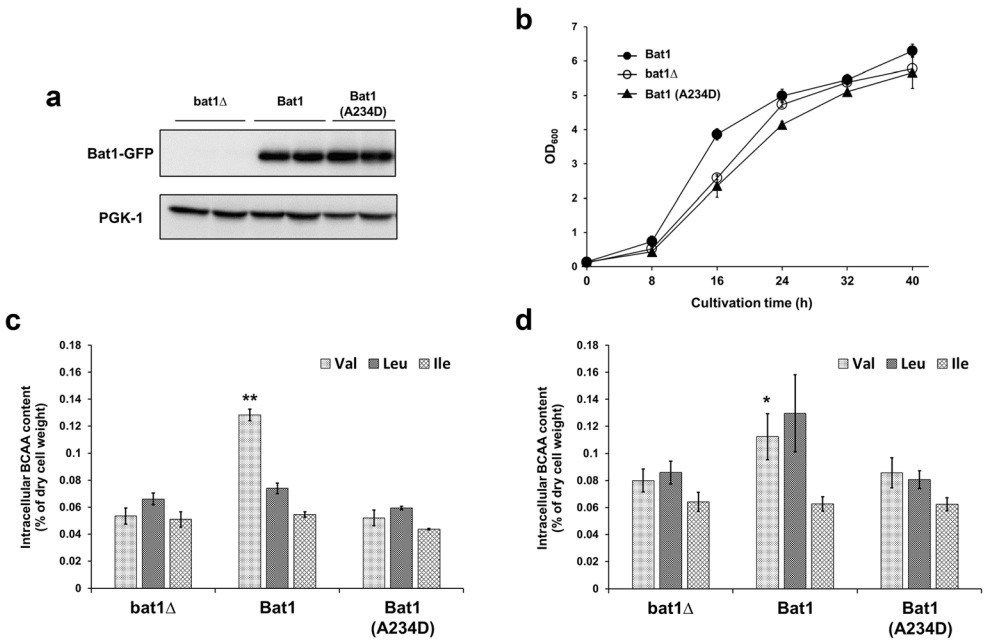

Fig. 2 Effects of amino acid substitution in Bat1 on yeast cell growth and BCAA production. The yeast transformants named bat1Δ, Bat1, and Bat1 (A234D) indicate BY4741*bat1*Δ (empty vector; EV), BY4741 (Bat1), and BY4741 (Bat1^{A234D}), respectively. **a** Western blotting analysis of the Bat1 variant expressing transformants. The two lanes underneath each genotype indicate protein extracts prepared from two independent colonies, grown in SD medium for 16 h. **b** Growth phenotype of bat1Δ (open circle), Bat1 (filled circle), and Bat1 (A234D) (filled triangle) were shown. Yeast cells were cultivated in SD medium. Each point represents the mean with standard deviations from three independent experiments. **c**, **d** Intracellular BCAA contents of yeast strains cultured in SD medium and collected at either the mid-log growth phase (**c**, 16 h) or the stationary growth phase (**d**, 32 h) are shown. Each point represents the mean with standard deviations from three independent experiments. Differences where $p < 0.05$(*) and $p < 0.01$(**) versus control (bat1Δ) were significant when verified by the non-repeated measured ANOVA followed by the Bonferroni correction

Effect of the A234D substitution in Bat1 on fusel alcohol production

In *S. cerevisiae* cells, the fusel alcohols isobutanol, isoamyl alcohol, and active amyl alcohol are synthesized from Val, Leu, and Ile, respectively, via the Ehrlich pathway in the cytoplasm (Hazelwood et al. 2008) (Fig. 1). Previous studies have reported that the production level of isobutanol increased in Bat1-defective strains (Hammer and Avalos 2017; Park et al. 2014). Based on these observations, we quantified the concentrations of these alcohols detected in the fermentation broth of yeast strains after cultivation for 3 days (Fig. 3). The amounts of isobutanol, isoamyl alcohol, and active amyl alcohol in BY4741 (Bat1) (7 ± 2, 6 ± 2, and 4 ± 1 ppm, respectively) were markedly lower than those in BY4741*bat1*Δ (EV) (34 ± 1, 43 ± 1 and 11 ± 0.5 ppm, respectively) and BY4741 ($Bat1^{A234D}$) (28 ± 7, 35 ± 8 and 9 ± 2 ppm, respectively). These results suggest that the A234D amino acid substitution in Bat1 contributes to an increase in fusel alcohol production in *S. cerevisiae*.

Effects of the A234D substitution in Bat1 on osmotic stress tolerance and fermentation ability

A previous study reported that BCAA derivatives [2-hydroxyisovalerate (HIV) from Val, 2-hydroxyisocaproate (HIC) from Leu and 2-hydroxy-3-methylvalerate (HMVA) from Ile] are involved in the osmotic stress response in *S. cerevisiae* cells (Shellhammer et al. 2017). That finding suggested that Bat1 function affects phenotypic differences under hyperosmotic conditions. To examine the effect of the A234D substitution in Bat1 on osmotic stress tolerance, we performed a spot test analysis of yeast cells under hyperosmotic conditions (Fig. 4a). In the presence of 2% glucose on SD agar medium, BY4741*bat1*Δ (EV) and BY4741 ($Bat1^{A234D}$) showed a growth delay over 1 day of incubation compared with BY4741 (Bat1), whereas those strains eventually exhibited the same levels of growth as BY4741 (Bat1) after 3 days of incubation (data not shown). Interestingly, BY4741 ($Bat1^{A234D}$) and BY4741*bat1*Δ (EV) showed higher sensitivities to high concentrations of glucose (10, 20, and 30%) than BY4741 (Bat1) after 3 days of incubation.

To further understand the function of Bat1 under the osmotic stress conditions, we tested the fermentation performance of yeast strains in SD liquid medium containing 2% glucose (the concentration of glucose in SD medium) or 20% glucose under static culture conditions for 14 days by measuring the production of carbon dioxide. As shown in Fig. 4b, all of the strains grown in normal SD medium (2% glucose) produced nearly equal amounts of carbon dioxide during fermentation. However, the rate of carbon dioxide emission was significantly decreased in BY4741*bat1*Δ (EV) and BY4741 ($Bat1^{A234D}$) compared with that in BY4741 (Bat1) in the presence of 20% glucose in the medium (Fig. 4c). These results indicate that Bat1 is involved in osmotic stress tolerance in yeast cells.

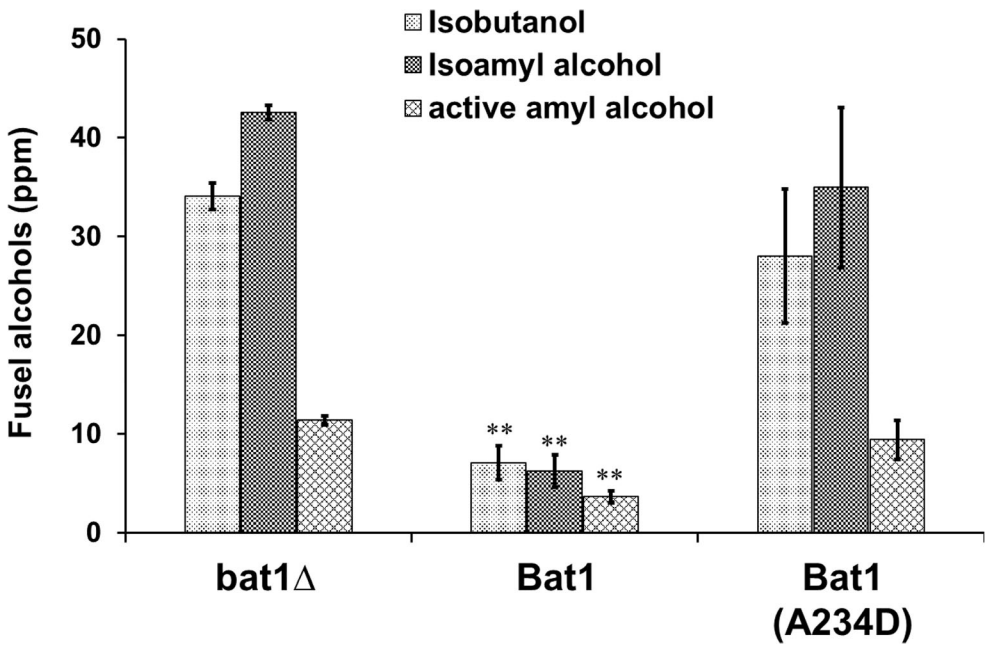

Fig. 3 Production of fusel alcohols in the yeast transformants. The fusel alcohol content, measured using GC-MS on 3-day-old yeast cultures grown in SD medium, is shown as means and standard deviations from three independent experiments. Differences where $p < 0.01$ (**) versus control (bat1Δ) were significant when verified by the Student's *t* test. The transformants named bat1Δ, Bat1, and Bat1 (A234D) indicate BY4741*bat1*Δ (empty vector; EV), BY4741 (Bat1), and BY4741 ($Bat1^{A234D}$), respectively. Differences where $p < 0.01$(**) versus control (bat1Δ) were significant when verified by the non-repeated measured ANOVA followed by the Bonferroni correction

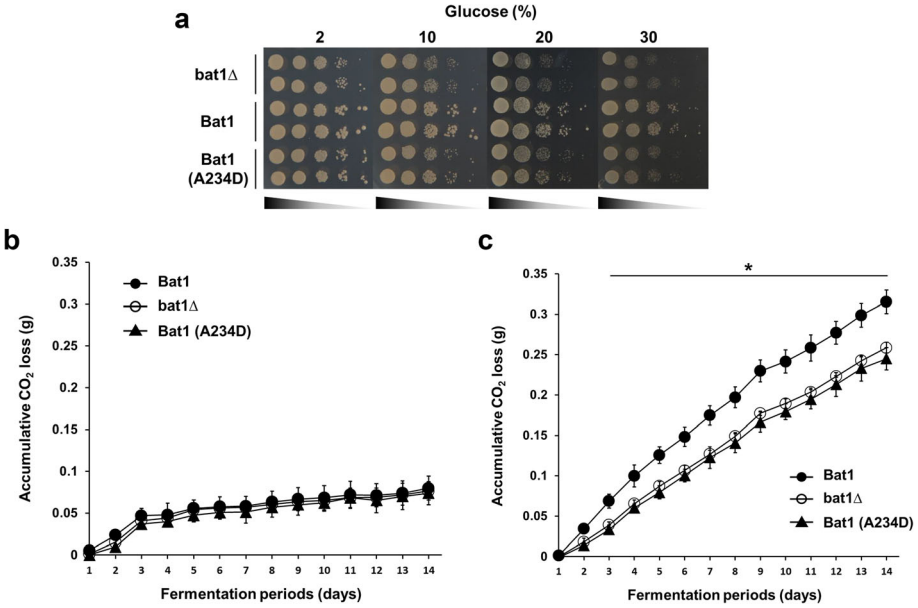

Fig. 4 Effect of hyperosmotic stress on yeast cells expressing Bat1. The transformants named bat1Δ, Bat1, and Bat1 (A234D) indicate BY4741*bat1*Δ (empty vector; EV), BY4741 (Bat1), and BY4741 (Bat1^{A234D}), respectively. **a** The growth phenotypes of bat1Δ, Bat1, and Bat1 (A234D) on SD plate medium containing 2, 10, 20, and 30% glucose for 3 days at 30 °C are shown. Fermentation abilities of bat1Δ (open circle), Bat1 (filled circle), and Bat1 (A234D) (filled triangle) in the presence of 2% (**b**) and 20% (**c**) glucose were shown. Accumulative CO_2 loss (g) was defined as a decrease in the weight of test tube, calculated by subtracting from that of day 1. Differences where $p < 0.05$ (*) versus control (bat1Δ) were significant when verified by the non-repeated measured ANOVA followed by the Bonferroni correction

Discussion

In *S. cerevisiae*, Bat1 plays an important role in the biosynthesis of BCAAs. In an accompanying study, we have found that an amino acid substitution from Ala to Asp at position 234 in Bat1 might affect the metabolism of BBCAs in beer yeast cells (Large et al. 2020). In the present study, to examine the effects of the A234D amino acid substitution in Bat1 on a variety of phenotypic traits, we generated a laboratory yeast strain (BY4741) that expresses the A234D variant Bat1.

Many phenotypes in yeast cells expressing the *bat1*^{A234D} gene were similar to those in the *bat1*Δ cells in terms of growth rate, intracellular contents of BCAAs, fusel alcohol production, and osmotic stress tolerance. The expression level of the Bat1 variant was comparable to that of the wild-type enzyme (Fig. 2a). These results strongly suggest that an Ala-to-Asp replacement at position 234 led to the dysfunction of the Bat1 protein without its degradation in yeast cells. Recent studies reported that amino acid substitutions in Bat1 at either an active site or substrate binding sites (K202R, R145E, Y71A, and K219A) led to a loss of enzymatic activity (Chen et al. 2012; Kingsbury et al. 2015). To investigate the mechanism responsible for the dysfunction caused by the A234D substitution in Bat1, we constructed a three-dimensional (3D) homology model of Bat1 based on the structure model of human BCAA aminotransferase (mBCAT) combined with KIV, which is a natural substrate of Bat1 and is converted into Val (PBD ID: 1KTA) (Fig. 5). The putative Bat1 3D model indicated that the amino acid residue at position 234 is located on the cleft of the α-helix (226–237) and far away (12.5 Å) from KIV when compared with the distances to the Arg145, Tyr71, and Lys219 residues from KIV (3.98, 2.96, and 2.90 Å, respectively). These results lead us to presume that neither Ala nor Asp at positon 234 interacts with the substrate binding or the catalytic sites directly. A previous study revealed that the aspartate residue (Asp-) with the helix dipole acts as a strong helix breaker by affecting the stability at positions throughout the helix—i.e., not only near the N-terminus but also near the C-terminus (Huyghues-Despointes et al. 1993). Moreover, Pace and Scholtz (1998) have reported that Asp- exhibited less helical propensity, while Ala which was the best residue in terms of promoting helix formation. Thus, it is possible that the substituted Asp (acidic amino acid) at position 234 interferes with the internal structure of Bat1 and then induces alterations in the Bat1 structure, especially in the structure of the α-helix, which could in turn lead to the dysfunction of Bat1. While the yeast BCATs' structures have not been determined to date, a previous study showed that several residues, such as Leu153, Gly154, Val155, Tyr70, Tyr141, Phe30, Thr240, and Ala314, in hBCATm stabilized the side chain of KIV (Yennawar et al. 2002). All of those residues are completely conserved in the Bat1 sequence, so that it is assumed that the yeast Bat1 metabolizes valine or KIV in the same manner as hBCATm. Although this is beyond the scope of this study, future studies of hBCATm or Bat1 structures would demonstrate the precise mechanism of transamination

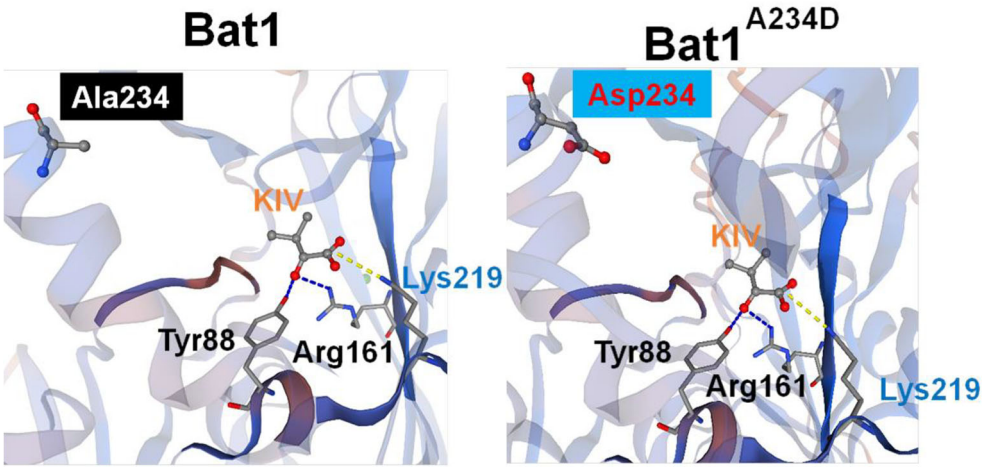

Fig. 5 Schematic diagrams of the predicted active center in Bat1 and Bat1^{A234D}. The conformational state of the wild-type Bat1 and the variant Bat1^{A234D} bound with α-ketoisovalerate (KIV) was scaffolded using the structure of the human BCAT, mBCAT (PDB ID code: 1KTA). A substituted residue in Bat1 (Ala to Asp at position 234), KIV, and amino acid residues involved in the catalysis of KIV is shown in the stick model

on Bat1. On the other hand, a previous study revealed that several proteins that are inactivated or lose their catalytic activity through amino acid substitutions showed other enzymatic properties or functions in yeast cells upon exposure to different stresses (Cantú et al. 2018). Yeast cells expressing the inactivated Bat2 were also shown to be tolerant to pH, SDS, and caffeine (Cantú et al. 2018). It should be noted that the amino acid sequences of Bat1 and Bat2 are highly homologous (77% identity). Considering these points, the Bat1 protein might be involved in the pathway of an unknown stress response. Therefore, further studies on the Bat1 structure may clarify the mechanism for dysfunction caused by the A234D substitution.

In the present study, the amount of fusel alcohols was greatly increased in strains lacking the *BAT1* gene and expressing the *bat1*^{A234D} gene compared with the wild-type strain. Despite several studies reporting that a deletion of the *BAT1* gene is effective for yielding an increase in isobutanol production in yeast cells (Park et al. 2014; Hammer and Avalos 2017), no previous report has made use of a Bat1 variant to overproduce fusel alcohols. It can be assumed that the elevated levels of α-keto acids (KIV, KIC, and KMV) were responsible for the increase in the productivity of fusel alcohols in the present study—namely, an excess of fusel alcohols was synthesized from KIV and KMV generated and overflowed from the mitochondria, which have a lower ability to produce BCAAs, to the cytoplasm. Although our results indicated that deficiency or inactivation of Bat1 was effective to generate higher amounts of fusel alcohols, it was previously reported that the isobutanol level was decreased or unchanged by applying this strategy (Ofuonye et al. 2013; Eden et al. 2001; Zhang et al. 2016). These inconsistent results indicate the presence of unknown mechanism(s) for regulating fusel alcohol production in *S. cerevisiae*. The elucidation of such mechanism(s) would be of much value to the field of industrial biofuel production.

In this study, *bat1*Δ cells in the logarithmic phase exhibited a significant decrease in Val content compared with the wild-type cells in the logarithmic phase, similarly to our previous data (Takpho et al. 2018). Although there were no significant difference in Leu content among the strains in the logarithmic phase, we found a decrease in Leu content in both *bat1*Δ and *bat1*^{A234D} cells compared with the wild-type cells in the stationary phase. It is well known that Leu **can be** synthesized directly from KIC by Bat2 in the cytoplasm and that the *BAT2* gene is mainly expressed in stationary phase in *S. cerevisiae* (Eden et al. 1996). It might be concluded that the Leu synthesis is upregulated during the stationary phase in yeast cells. Indeed, all of the strains showed a tendency toward the increased Leu content in the stationary phase, in comparison with the logarithmic phase. However, the wild-type strain showed a 1.8-fold increase in Leu content in the stationary phase compared with the logarithmic phase, versus a 1.3-fold increase in the other strains. These results suggest that Bat1 highly contributes to the Leu production from KIC, which is a metabolite of KIV. Moreover, no significant differences in intracellular Ile contents among the strains were observed, suggesting that Bat1 does not contribute to the Ile production from KMV. A previous report revealed that Bat1 has a substrate preference for KIC (converted into Leu) over KMV (converted into Ile) as a substrate (Colón et al. 2011). In addition, the BCATs expression and bidirectional enzymatic activity are controlled in the presence of different kinds of nitrogen sources in the medium (Colón et al. 2011: González et al. 2017). These findings suggest the presence of unknown regulation mechanisms of BCATs in *S. cerevisiae*. Attempts have been made to clarify the metabolic regulation in the stationary phase (Zakrajšek et al. 2011: Aris et al. 2012: Mukai et al. 2019). Normally, the fermentation process proceeds over a long period of time—namely, days to weeks. Further study is

needed to reveal the functions of Bat1 and Bat2 in both the stationary and logarithmic phases.

Large et al. investigated the effects of the *BAT1* gene mutation leading to the A234D amino acid substitution in Wyeast 1056 on the production of aromatic components (Large et al. 2020). These aromatic components, which consist of higher alcohols and esters, are key elements produced by yeast cells, which will determine the final quality of beer (Pires et al. 2014). In the present study, we focused on the effect of the A234D replacement in Bat1 and uncovered sufficient evidence that the A234D variant Bat1 is a catalytically inactive form and is responsible for producing a higher amount of fusel alcohols. It was also found that *bat1*Δ and *bat1*^{A234D} cells were sensitive to osmotic stress and exhibited reduced fermentation ability in the presence of 20% glucose. BCATs are reported as necessary for a full response to osmotic stress (Shellhammer et al. 2017). In addition, a dysfunctional BCAT mutation perturbed TCA cycle intermediate levels, consistent with a TCA cycle block, and resulted in low ATP levels, activation of AMP-activated protein kinase (AMPK), and TORC1 inhibition (Kingsbury et al. 2015). Several other studies also demonstrated that the *BAT1* gene deletion led to an impairment of cell growth in minimal medium (Kispal et al. 1996: Colón et al. 2011: Park et al. 2014: Takpho et al. 2018), indicating that a deficiency of the *BAT1* gene has a negative effect on yeast cell growth. In the study by Large et al., the *BAT1* gene mutation was discovered in a heterozygous tetraploid industrial beer yeast strain and possessed three wild-type and one mutant *BAT1* allele (Large et al. 2020). Thus, we propose that the *bat1*^{A234D} allele induces a haploinsufficient phenotype and contributes to an increase of volatile flavor compounds during beer fermentation. As beer fermentation is traditionally performed with the brewer's worts, which is composed of about 9–12% of fermentable sugars such as maltose, maltotriose, and glucose, we suspect that the sugar content of the wort puts an osmotic pressure on the yeast cells that is exacerbated by the lack of a functional allele (Patel and Ingledew 1973; Huuskonen et al. 2010). In addition, for an increase in production capacity, many large breweries use the so-called high-gravity worts containing total sugar contents of around 12–20% (Huuskonen et al. 2010). Even though the brewing under the high osmotic conditions likely affects the fermentation kinetics of the strain due to the *bat1*^{A234D} allele, they likely retain the ability to robustly ferment worts via the contribution of the other wild-type *BAT1* alleles, thereby successfully achieving an increase in fusel alcohols as volatile flavor components. We suggest that further work into the relationship of intracellular metabolism and osmotic stress will aid in the development of more robust beer brewing strains.

Author contributions MD and HT conceived the study and designed the experiments. JK performed the experiments. JK, YT, MO, CL, and HT analyzed the data. JK, YT, MO, and HT wrote the manuscript. All authors reviewed and approved the final version of manuscript.

Funding information This work was supported by a Grant-in-Aid for Research Activity start-up (19K21144) from the Japan Society for the Promotion of Science (JSPS) to YT and a grant from the Project of the NARO Bio-oriented Technology Research Advancement Institution (Research program on development of innovative technology) (30017B) to HT. CL was supported by NIH grant T32 HG00035. MD and CL were supported by the National Science Foundation under grant 1516330 and under Cooperative Agreement No. DBI-0939454. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. The research of MD was supported in part by a Faculty Scholar grant from the Howard Hughes Medical Institute.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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