High-level production of valine by expression of the feedback inhibition-insensitive acetohydroxyacid synthase in *Saccharomyces cerevisiae*

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**Abstract**

Valine, which is one of the branched-chain amino acids (BCAAs) essential for humans, is widely used in animal feed, dietary supplements and pharmaceuticals. At the commercial level, valine is usually produced by bacterial fermentation from glucose. However, valine biosynthesis can also proceed in the yeast *Saccharomyces cerevisiae*, which is a useful microorganism in fermentation industry. In *S. cerevisiae*, valine biosynthesis is regulated by valine itself via the feedback inhibition of acetohydroxyacid synthase (AHAS), which consists of two subunits, the catalytic subunit Ilv2 and the regulatory subunit Ilv6. In this study, to improve the valine productivity of yeast cells, we constructed several variants of Ilv6 by introducing amino acid substitutions based on a protein sequence comparison with the AHAS regulatory subunit of *E. coli*. Among them, we found that the Asn86Ala, Gly89Asp and
Asn104Ala variants resulted in approximately 4-fold higher intracellular valine contents compared with those in cells with the wild-type Ilv6. The computational analysis of Ilv6 predicted that Asn86, Gly89 and Asn104 are located in the vicinity of a valine-binding site, suggesting that amino acid substitutions at these positions induce conformational change of the valine-binding site. To test the effects of these variants on AHAS activity, both recombinant Ilv2 and Ilv6 were purified and reconstituted in vitro. The Ilv6 variants were much less sensitive to feedback inhibition by valine than the wild-type Ilv6. Only a portion of the amino acid changes identified in the *E. coli* AHAS regulatory subunit IlvH enhanced the valine synthesis, suggesting structural and/or functional differences between the *S. cerevisiae* and *E. coli* AHAS regulatory subunits. It should also be noted that these amino acid substitutions did not affect the intracellular pools of the other BCAAs, leucine and isoleucine. The approach described here could be a practical method for the development of industrial yeast strains with high-level production of valine or isobutanol.

1. Introduction

Valine is one of the branched-chain amino acids (BCAAs) essential for humans, along with leucine and isoleucine, and valine deficiency has been linked to several human diseases (Hutchison et al., 1983; Burrage et al., 2014). Since valine is a hydrophobic amino acid, it often forms the helical structures within interior proteins. Valine is included in various commercial products, such as animal feed, human dietary supplementaries, pharmaceuticals and cosmetics. In addition, derivatives of valine can be used as a substrate for antibiotics and antivirals as well as in herbicide production via the chemical synthon processes. Many industrial applications are currently utilizing valine (Stoner et al., 2000; Eggeling et al., 2001), and thus the global demand for valine – especially food-grade valine – has grown in the past few years (Cheiljedang, 2013). Therefore, an intensive study of valine metabolism will be important for gaining a better
understanding of the metabolic regulation of valine, which may be applicable for both industrial
applications and medical research. Generally, valine is biosynthesized by plants, algae, fungi,
bacteria and archaea, but not in animals. In addition, biotechnology processes have been applied
to industrial valine production in order to meet consumer demand. Microorganisms appear to
be promising hosts for large-scale production, since they can provide high productivity, rapid
growth rates, the ability to utilize several substrates and climate-independent culture processes.
For many years, valine has been commercially produced from bacteria, such as
*Corynebacterium glutamicum* and *Escherichia coli*, using several metabolic engineering
approaches to improve the productivity (Park et al., 2007; Park and Lee, 2010).

The yeast *Saccharomyces cerevisiae* has received an attention as a suitable host for food-
and pharmaceutical-grade products due to its safety and generally recognized as safe (GRAS)
status. Yeast cells contain high concentrations of protein, RNA, lipids, amino acids and vitamins
that can be utilized as nutrient-rich dietary supplements for animals and humans. In addition,
yeast extract is a product of the enzymatic digestion of yeast cells, and is currently applied in
several industries – e.g., as a flavoring agent in canned foods and packaged vitamin supplements,
and as a nitrogen source for microorganisms in scientific experiments (York and Ingram, 1996;
Chae et al., 2001). Based on their reliability and safety in food production, the development of
novel yeast strains that overproduce valine may make an important contribution to food-related
industries. Another applicable aspect of valine biosynthesis in *S. cerevisiae* is isobutanol
production (Hazelwood et. al., 2008). Isobutanol, which is a second-generation biofuel, is
produced from valine degradation in the cytosol, and therefore the enzymes associated with
valine biosynthesis have been engineered to be relocalized from mitochondria to cytosol (Brat
et. al., 2012) (Fig. 1). Based on a screening of butanol-tolerant microorganisms, *S. cerevisiae*
has been categorized as a better candidate for industrial butanol production than *E. coli*
(Knoshaug and Zhang, 2009).
Valine in *S. cerevisiae* is primarily biosynthesized in the mitochondria from two pyruvate molecules by mitochondrial enzymes, acetohydroxyacid synthase (AHAS; Ilv2/Ilv6; also referred to as acetalactate synthase), acetohydroxyacid isomeroreductase (AHAIR; Ilv5), dihydroxyacid dehydratase (DHAD; Ilv3), to produce the key intermediate, α-ketoisovalerate (KIV) (Fig. 1). By the assistance of unknown keto-acid transporters, KIV is partially transported to the cytosol. The final step of valine biosynthesis occurs in both mitochondria and cytosol via the mitochondrial and cytosolic BCAA aminotranseferase Bat1 and Bat2, respectively (McCourt and Duggleby, 2005). AHAS is the rate-limiting enzyme for BCAA biosynthesis and catalyzes the first step of BCAA biosynthesis by converting pyruvate molecules to 2-acetolactate (Umbarger and Brown, 1958). This enzyme consists of two subunits, the catalytic subunit (Ilv2) and the regulatory subunit (Ilv6). The expression of the *ILV2* and *ILV6* genes is regulated by the general amino acid synthesis activating transcription factor Gcn4 (Magee and Hereford, 1968; Xia and Rank, 1988; Pang and Duggleby, 1999). In addition, the enzymatic activity of AHAS is negatively regulated by valine via the process known as feedback inhibition. A previous study revealed that AHAS activity of the catalytic subunit alone is unaffected by high concentrations of BCAA, while the enzymatic activity of the reconstituted catalytic subunit and regulatory subunit were inhibited by valine, indicating a significant role of the regulatory subunit in feedback regulation (Cullin et al., 1996; Hill et al., 1997; Pang and Duggleby, 2001). Moreover, other valine derivatives, such as N-acetylvaline, N-methylvaline and valinamide, had no effect on the AHAS activity, suggesting that valine binds to the regulatory subunit in a specific manner. Taken together, these results indicate that the AHAS regulatory subunit Ilv6 is essential for the feedback inhibition by valine and for the full enzymatic activity (Pang and Duggleby, 2001).
In this study, we focused on the metabolic regulation of valine, particularly the regulatory subunit of AHAS (Ilv6), in *S. cerevisiae*. We successfully constructed yeast strains that significantly increase cellular valine levels by amino acid substitutions in Ilv6 based on a protein sequence comparison with the AHAS regulatory subunit of *E. coli*. It was also found that the Asn86Ala, Gly89Asp and Asn104His variants were less sensitive to feedback inhibition by valine than the wild-type Ilv6. Finally, we discuss the mechanism of valine overproduction by the above-described Ilv6 variants. This study reports the removal of feedback inhibition of AHAS activity, leading to valine overproduction in yeast cells.

2. Materials and methods
2.1. Strains and culture media

*S. cerevisiae* BY4741 (derived from S288c) was used in this study. Yeast cells were grown in a nutrient-rich YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) for routine culture. A synthetic dextrose (SD) minimal medium (1.7 g/L yeast nitrogen base without amino acid and ammonium sulfate, 5 g/L ammonium sulfate and 20 g/L glucose, pH 6.0) was used for cells harboring pRS416 and pRS415-CgHIS3MET15. The valine toxic analog DL-norvaline (Chem-Impex International, Inc., Wood Dale, IL, USA) was supplemented at a concentration of 1 mg/mL or 10 mg/mL for the screening of valine-accumulating cells. An *E. coli* DH5α was used for plasmid propagation and the bacterial transformation was carried out by the method for high efficiency transformation (Inoue et al., 1990). Bacterial cells were cultured in Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) contained 100 µg/mL of ampicillin. If necessary, 2% agar was added to solidify the medium. In case of bacterial cells harboring pDONR221, cells were cultured in LB medium containing 50 µg/mL of kanamycin. For protein expression, *E. coli* Rosetta™ (DE3) pLysS cells were cultured in LB medium containing 100 µg/mL ampicillin and 30 µg/mL chloramphenicol (Wako Pure Chemical Industries, Tokyo, Japan).

2.2. Plasmid construction

Yeast centromere plasmid (YCp) pRS416 was employed for construction of the *E. coli*-S. cerevisiae shuttle vectors in this study. The *ILV2* and *ILV6* genes were amplified from *S. cerevisiae* BY4741 genomic DNA by KOD FX DNA polymerase (Toyobo, Osaka, Japan) using corresponding primers (Supplementary Table S1), and subsequently cut and inserted to pRS416 at the *Sma*I and *Not*I recognition sites. Site-direct mutagenesis was used to introduce several amino acid substitutions, Asn86Ala, Gly89Asp, Asn104His, Ile255Ala/Arg and Met276Ala/Met, into the Ilv6 protein. Each mutation was introduced into the *ILV6* gene on
pRS416 using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) and mutagenic primer pairs (Supplementary Table S1). PCR products were then digested with DpnI before introduction into *E. coli* DH5α cells as the same manner as described by Inoue et al. (1990). Plasmid pRS416, which expresses the Ilv6 variants, was further confirmed by DNA sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). Plasmid pRS415-CgHIS3MET15 (constructed by S. Morigasaki) was co-transformed with the constructed pRS416-based plasmids to complement the auxotrophic phenotype of *S. cerevisiae* BY4741 using high-efficiency transformation method (Burke et al., 2000). The plasmids for protein expression were constructed by Gateway® cloning technology (Invitrogen, Carlsbad, CA, USA), based on the site-specific recombination system. The wild-type *ILV2*, *ILV6* or mutant *ILV6* genes were amplified and all PCR products were tagged with *attB1* and *attB2* sequences. PCR products were purified and inserted to the pDONR22 using BP clonase™ II (Invitrogen, USA). The BP clonase mixture was subsequently introduced to *E. coli* DH5α cells and confirmed by DNA sequencing. Desired transformants were subjected to plasmid DNA extraction and then incubated with protein expression plasmid, pET-53-DEST using LR clonase™ II (Invitrogen, Carlsbad, CA, USA) and introduced into *E. coli* DH5α cells.

2.3. Spot test for DL-norvaline resistance

*S. cerevisiae* cells harboring pRS416-ILV6 series were cultured in 5 mL of SD medium. After overnight incubation at 30 °C with rotary shaking, cells corresponding to an OD₆₀₀ of 10 were collected, washed twice with distilled water and suspended in 1 mL of water. Subsequently, 10-fold serial dilutions of yeast cells were prepared, 2.5 µL of each dilution was dropped on SD agar plates containing 1 mg/mL or 10 mg/mL of DL-norvaline and incubated at 30 °C for 3 days.

2.4. Measurements of intracellular amino acid content
S. cerevisiae cells were pre-cultured in 5 ml of SD medium for 16 h at 30 °C and transferred to 25 mL of SD medium for cultivation at 30 °C with shaking at 200 rpm. Yeast cells corresponding to an OD600 of 10 were collected, washed twice with distilled water and suspended in 500 µL of distilled water. Intracellular amino acids in cell suspension were extracted by boiling for 15 min at 100 °C. Cell debris was centrifuged at 13,000 × g for 1 min and subsequently omitted by filtration using 0.2 µm syringe filter (mdiTM, Ambala Cantt, India). Samples were subjected to analysis with an amino acid analyzer (AminoTacTM JL500/V; JEOL, Tokyo, Japan). Intracellular amino acid concentrations were expressed as a percentage of dry cell weight.

2.5. Protein expression

E. coli Rosetta™ (DE3) pLysS cells with pET-53-DEST harboring ILV2 and ILV6 series were pre-cultured at 30 ºC for overnight in LB medium containing 100 µg/mL ampicillin and 30 µg/mL chloramphenicol. The main culture was performed by inoculating cells into a 300 mL flask at the initial OD600 of 0.008 and incubating at 37 ºC until OD600 was reached to 0.3-0.4. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 100 µM and cultured at 18 ºC for 18 h with a high aeration rate of 250 rpm. Cells were harvested by placed on ice for 5 min and centrifuged at 4 ºC for 10 min at 5,000 × g. Cell pellets were resuspended in ice-cold lysis buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.9) containing protease inhibitors.

2.6. Protein purification

All reagents were stored at 4 ºC. Lysozyme (10 mg/g cells) was added to the mixture above. Cell lysate mixture was subjected to sonication (duty cycle 50%, output = 2, 30 sec/on ice 1 min for 5 cycles) and subsequently centrifuged at 10,000 × g for 20 min at 4 ºC, then filtrated
by 0.45 µM diameter filter. Crude protein was purified by His-accept column (Nacalai Tesque, Kyoto, Japan) in the same manner that described in a previous study (Pang and Diggleby, 2001). Purified-proteins were collected in 1.5 mL tubes containing 100 mM dithiothreitol (DTT) and 0.2 M potassium phosphate (pH 6.0) at the final concentration was immediately added to the purified Ilv2 to maintain the catalytic activity. According to the previous study, the purified proteins can be stored as follows. The purified Ilv2 was stored in the elution buffer contains 10 µM FAD and 20% glycerol. For the purified Ilv6, 0.1 M potassium phosphate (pH 7.0) and 20% glycerol were added to the buffer; small aliquots were kept at -80 ºC. It should be noted that the catalytic activity was reduced by 80% when the protein was stored overnight at 4 ºC (Pang and Diggleby, 1999). Proteins were quantified using Bio-Rad Protein Assay (Hercules, CA, USA) and subjected to SDS-polyacrylamide gel electrophoresis.

2.7. Reconstitution of purified AHAS subunits and Assay of AHAS activity

Reconstitution of purified Ilv2 and Ilv6 (wild-type and variants) was carried out as described before (Pang and Diggleby, 1999; 2001). The reaction was performed at 30 ºC in the mixture contained 200 mM pyruvate, 1 mM ThDP, 10 mM MgCl₂ and 10 µM FAD in 1 M potassium phosphate buffer (pH 7.0). The enzyme was pre-incubated at 30 ºC for 15 min in 225 µL of the reaction mixture without pyruvate. Then, 25 µL of 2 M pyruvate was added to the reaction mixture, and further incubated for 20 min at 30 ºC. In case of the experiment for valine inhibition, various concentrations of L-valine ranging from 0.25 mM to 1 mM were added before the incubation. Thirty-five µL of 50% (v/v) sulphuric acid was added to stop the reaction, and further incubated at 60 ºC for 15 min to facilitate the conversion of α-acetolactate into acetoin. AHAS activity was measured by single-point colorimetry, as described by Singh et al. (1988). The color was developed by adding 400 µL of 0.5 % (w/v) creatine and 5% (w/v) α-naphthol (in 4 M NaOH), and then incubated at 60 ºC for 15 min. The color mixture was
subsequently measured with a spectrophotometer (U-1100 Spectrophotometer, Hitachi, Tokyo, Japan) at 525 nm, using the data in a reaction without enzyme as a blank. Standard acetoin was varied from 0.2 µM to 20 µM. One unit of activity was defined as the amount of enzyme required to produce 1 µmol of α-acetolactate per min under the above conditions. Specific activity was expressed as enzyme units per mg of catalytic subunit as determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. Design of amino acid substitutions of the AHAS regulatory subunit (Ilv6) for valine overproduction

_E. coli_ and _S. cerevisiae_ have similar mechanisms for regulating valine biosynthesis, in which AHAS activity is subjected to feedback inhibition by valine. Therefore, the AHAS regulatory subunit of _E. coli_ (IlvH; AHAS III) was used as a model in this study. A previous study in _E. coli_ demonstrated that several amino acid substitutions in the IlvH protein confer resistance to valine feedback inhibition on AHAS activity (Mendel et al., 2001; Kaplun et al., 2006). The protein sequence alignment between the _E. coli_ IlvH and the _S. cerevisiae_ Ilv6 revealed a comparable structure at the ACT (aspartate kinase, chorismate mutase and TyrA) domain and the ALS_ss_C (acetohydroxyacid synthase, small subunit and C-terminal) domain (Fig. 2A). According to this information, we hypothesized that the amino acid residues involved in feedback inhibition by valine are Asn86, Gly89, Asn104, lle255 and Met276 of Ilv6 in _S. cerevisiae_, which correspond to Asn11, Gly14, Asn29, Leu131 and Val153 of IlvH in _E. coli_, respectively (Fig. 2B). It was notable that Asn11, Gly14 and Asn29 are conserved between the _E. coli_ IlvH and the _S. cerevisiae_ Ilv6. Three mutations, leading to the Asn11Ala, Gly14Asp and Asn29His substitutions, were isolated from the spontaneous ilvH mutants, while the Leu131Ala/Arg and Val153Ala/Asp substitutions were identified by PCR-based random
mutagenesis at the C-terminal region of IlvH (Mendel et al., 2001; Kaplun et al., 2006). Thus, amino acid substitutions of the *S. cerevisiae* Ilv6 that correspond to the previously reported mutations of the *E. coli* ilvH were introduced as follows: Asn86Ala, Gly89Asp, Asn104His, Ile255Ala/Arg and Met276Ala/Asp.

### B

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**Fig. 2.** The amino acid sequence alignment of AHAS regulatory subunits, the *E. coli* IlvH and the *S. cerevisiae* Ilv6. (A) The schematic representation of the *E. coli* IlvH and the *S. cerevisiae* Ilv6. The ACT domain and the ALS ss C domain are orange and yellow, respectively. (B) The amino acid sequence alignment of the *E. coli* IlvH and the *S. cerevisiae* Ilv6. Residues N11, G14, N29, I131 and V153 in IlvH, where substitutions resulted in the resistance to feedback inhibition by valine (Kaplun et al., 2006), are indicated by red in orange and yellow box. The amino acid substitutions in Ilv6 are indicated by arrowhead below the sequences. The numbers are residue numbers. Identical and similar amino acids in the two proteins are indicated by the same residue and 1, respectively. Dashes indicate the absence of corresponding amino acid residues at the positions.

### 3.2. Effects of amino acid substitutions of the AHAS regulatory subunit (Ilv6) on DL-norvaline-resistance

To identify the mutations in *ILV6* that abrogate feedback inhibition by valine, we tested the growth phenotypes of yeast cells expressing the wild-type and variant Ilv6 on SD medium containing 1 mg/mL or 10 mg/mL DL-norvaline, which is a toxic valine analogue. It was
expected that valine accumulation would alleviate the cytotoxicity of DL-norvaline, which
might be incorporated into proteins competitively with valine. As shown in Fig. 3A, the
Asn86Ala, Gly89Asp and Asn104His variants of Ilv6 remarkably increased DL-norvaline
resistance of yeast cells as compared to the wild-type Ilv6, indicating that the DL-norvaline-
resistant growth phenotype of the variants was dominant. This result was consistent with
previous studies in *E. coli* (Mendel et al., 2001; Kaplun et al., 2006), suggesting that these
amino acid substitutions in Ilv6 cause valine accumulation in yeast cells. However, the
Ile255Ala/Arg and Met276Ala/Asp variants did not clearly show the DL-norvaline resistance.
The amino acid substitutions in the ALS_ss_C domain of Ilv6 exhibited a different effect from
the corresponding mutations in the *E. coli ilvH*. Based on these results, it was found that the
Asn86Ala, Gly89Asp and Asn104His substitutions on Ilv6 are responsible for the DL-norvaline-
resistant phenotype in *S. cerevisiae*.

The predicted structure of the yeast Ilv6 (based on PSI-blast and PyMOL) clearly showed
that Asn86, Gly89 and Asn104 are located in the vicinity of the valine-binding site in the ACT
domain (Fig. 4). In addition, we noted that Asn104 binds to valine upon the dimer assembly.
Since the binding of valine to these residues has not been well studied, we further investigated
the effects of different amino acid substitutions at positions 86, 89 and 104 on the DL-norvaline
resistance, based on the side-chain polarity, charge and hydropathy index (Fig. 3B). The results
showed that any substitutions at these positions increased the DL-norvaline resistance to the
same degree. In addition, the combination of Asn86Ala or Gly89Asp with Asn104His did not
show any clear additive effects.
Fig. 3. Growth phenotypes on D,L-norvaline-containing medium of yeast cells expressing IIV6. Cell suspensions with 10-fold serial dilutions were dropped (2.5 μL each) on SD agar medium, which was supplemented with 0.1% allantoin instead of ammonium sulfate as the sole nitrogen source, in the absence or presence of 10 mg/mL D,L-norvaline, then incubated at 30 °C for 3 days.
3.3. Effects of amino acid substitutions of the AHAS regulatory subunit (Ilv6) on intracellular valine contents

In general, greater resistance to DL-norvaline reflects a higher level of intracellular valine (Park et al., 2007; 2011). To examine whether the DL-norvaline-resistant phenotype is associated with intracellular valine accumulation, yeast cells expressing the wild-type and variant Ilv6 were cultivated in SD liquid medium, and the cellular valine levels were examined (Fig. 5). As we expected, yeast cells expressing the Asn86Ala, Gly89Asp and Asn104His variants of Ilv6 showed an approximately 4-fold increase in the valine content compared with that of the wild-type Ilv6. The additive effect of substitution, Asn86Ala/Asn104His and Gly89Asp/Asn104His, on the valine content was not observed in agreement with the result of the spot test on DL-norvaline-containing plates (Fig. 3B). Unexpectedly, the combination of three substitutions, Asn86Ala, Gly89Asp and Asn104His, significantly decreased the intracellular valine level to that of the wild-type cells via unknown mechanisms. In contrast, the Ilv6 variants did not have any significant effects on the intracellular level of the other
BCAAs, leucine and isoleucine (Fig. 5). Thus, it was concluded that the amino acid substitutions at the positions 86, 89 and 104 of Ilv6 specifically elevated the intracellular valine contents, leading to the DL-norvaline-resistant phenotype of yeast cells.

![Graph showing intracellular branched-chain amino acid contents](image)

**Fig. 5. Intracellular branched-chain amino acid contents.** Yeast cells were grown in SD medium (pH 6.0) at 30°C and collected at log-phase (15 h after inoculation). White, light gray and dark gray bars represent intracellular valine, leucine and isoleucine contents, respectively. The values are the means and standard deviations of three independent experiments. Asterisks indicate statistically significant differences in comparison to the control (wild-type cells (BY4741) with the empty vector) (Tukey’s test, p < 0.05).

3.4. Effects of amino acid substitutions of the AHAS regulatory subunit (Ilv6) on enzymatic properties

Since the Ilv6 variants, Asn86Ala, Gly89Asp and Asn104Ala, exhibited the DL-norvaline-resistant phenotype and higher intracellular valine contents, we hypothesized that these amino acid substitutions induce some conformational change of the valine-binding site,
leading to reduction of the valine-binding affinity. In order to examine this hypothesis, the AHAS activity was measured using the in vitro reconstituted AHAS, which are comprised of Ilv2 (the catalytic subunit responsible for catalytic activity) and Ilv6 (the regulatory subunit for modulating the catalytic subunit) (Pang and Diggleby, 1999). To prepare the recombinant proteins, Ilv2 and Ilv6 tagged with 6x His at the amino terminus were expressed in the E. coli Rosetta™ (DE3) pLysS strain. However, due to the insolubilization of the recombinant proteins in the cell, the putative transit peptides at the amino terminus of Ilv2 (54 amino acids) and Ilv6 (40 amino acids) were removed to increase the protein solubility, since they are not required for bacterial protein expression (Pang and Duggleby, 1999). As a result, the solubility of Ilv2 and Ilv6 without transit peptides was remarkably improved for further purification (data not shown).

The AHAS activities of purified-recombinant Ilv2 and Ilv6 were determined based on the level of acetoin, which is produced from acetyl-CoA, the product from the AHAS reaction. The AHAS activity of purified Ilv2 was 34.1 U/mg protein, while no AHAS activity was detected in the reaction containing purified Ilv6 alone (data not shown). In S. cerevisiae, the ILV2 gene encoding the catalytic subunit Ilv2 was first identified and cloned by complementation of the ilv2 mutation, which is deficient in the AHAS activity (Poliana, 1984). In addition, the regulatory subunit of AHAS was later identified based on the similarity of bacterial AHAS (Oliver et al., 1992). Ilv6 is not involved in the catalytic activity; however, it is responsible for the feedback inhibition by valine, since the AHAS activity in Δilv6 cells was no longer inhibited by valine (Cullin et al., 1996). With an increase in the protein amount of Ilv6, AHAS activity was significantly increased until the saturation point, where the concentration of Ilv6 was around 100-fold that of Ilv2 (Pang and Duggleby, 1999), suggesting that Ilv6 is required to achieve the full level of AHAS activity. In this study, high AHAS activity was observed in the reaction containing Ilv2 and Ilv6 in a ratio of 1:100 (125 ± 6 U/mg of protein for the wild-type), whereas 34 ± 4 U/mg was detected in the reaction containing only Ilv2. Based on these
preliminary results, it was suggested that purified Ilv2 and Ilv6 were successfully reconstituted in this experiment.

We next analyzed feedback inhibition of the AHAS activity by valine (Fig. 6). Enzymatic assays without valine in the reaction mixture revealed that the AHAS activity reconstituted by the Ilv6 variants (156 ± 11 U/mg for Asn86Ala, 145 ± 8 U/mg for Gly89Asp and 152 ± 6 U/mg Asn104His) was slightly higher than that from the wild-type Ilv6 (125 ± 6 U/mg). However, the activity of AHAS reconstituted by the wild-type Ilv6 and Ilv2 was drastically inhibited by 0.2 mM valine and consequently decreased when the valine concentration was increased, indicating that the AHAS activity of *S. cerevisiae* cells is subject to feedback inhibition by valine; that is, AHAS is the rate-limiting enzyme in the valine biosynthesis of *S. cerevisiae* cells. In contrast, the level of AHAS activity from the Ilv6 variants (Asn86Ala, Gly89Asp and Asn104His) and wild-type Ilv2 was approximately 70-80% even in the presence of 1.0 mM valine. These results indicate that diminished sensitivity to valine feedback inhibition in AHAS causes valine accumulation. It was also noted that the activity of AHAS reconstituted by wild-type Ilv6 and Ilv2 in the presence of 1.0 mM valine (32 ± 5 U/mg) was decreased to a similar level as observed in single Ilv2 (34 ± 4 U/mg). These results were also consistent with the previous report that AHAS activity is inhibited by valine via the regulatory subunit Ilv6 (Cullin et al., 1996).
In bacteria, valine is produced from two pyruvate molecules by four enzymes, AHAS, AHAIR, DHAD and transaminase B (Oldiges et al., 2014). The key enzyme of valine biosynthesis is AHAS, which is subjected to the feedback inhibition by valine (Pang and Duggleby, 1999). However, the feedback inhibition of AHAS by valine in *C. glutamicum* is weaker than that in *E. coli*: 50% of the maximum AHAS activity in *C. glutamicum* remains even in the presence of 10 mM valine (Elišáková et al., 2005), whereas 80% of the maximum AHAS activity in *E. coli* is inhibited by 4.8 µM valine (Mendel et al., 2001). Therefore, the *E. coli* AHAS is a suitable model for the study of feedback inhibition by valine. There are three AHAS isozymes found in *E. coli* (AHAS I, II and III), whereas only one isozyme exists in *S. cerevisiae*. The enzymatic activities of *E. coli* AHAS I and AHAS III are inhibited in the presence of valine via the regulatory subunits, IlvN and IlvH, which are responsible for valine
feedback inhibition. Nevertheless, the protein alignment of *E. coli* IlvN is unique and rather
different from that of yeast Ilv6 unlike that of the *E. coli* IlvH (PSI-Blast: Altschul et al., 1997).
Accordingly, we employed the regulatory subunit of *E. coli* AHASIII (IlvH) as a model of the
regulatory mechanism of valine biosynthesis in *S. cerevisiae*.

Based on their protein sequence alignment, the *E. coli* IlvH and the *S. cerevisiae* Ilv6
showed similarly conserved regions at the ACT domain and the ALS_ss_C domain (Fig. 2).
Here, the intracellular valine increased approximately 4-fold when the Ilv6 variants, Asn86Ala,
Gly89Asp and Asn104His, in which amino acid substitutions are located at the ACT domain,
were expressed (Fig. 5). On the other hand, amino acid changes at the ALS_ss_C domain,
Ile255Ala, Ile255Arg, Met276Ala and Met276Asp, did not have any effects on the valine
feedback inhibition. In general, the ACT domain is responsible for the binding to ligands or
small molecules for the regulation of BCAAs biosynthesis, whereas the ALS_ss_C domain was
reported to be involved in binding of other compounds, such as MgATP (Duggleby, 1997; Pang
and Duggleby, 2001). In *E. coli*, the residues Asn11, Gly14 and Asn29 in IlvH are highly
conserved among several microorganisms, including *S. cerevisiae*, suggesting that the amino
acid changes at these three positions could lead to the removal of valine feedback inhibition. In
*S. cerevisiae*, Asn86 and Asn104 are predicted to locate at the putative valine-binding site (Fig.
4) (Phyre2: Kelley et al., 2015); hence, amino acid substitution at the valine-binding site
probably affects the conformation of this pocket. The valine-inhibitory experiments also
showed that the reconstituted-AHAS with the wild-type Ilv6 was sensitive to feedback
inhibition by 0.2 mM of valine, and the AHAS activity was decreased to the basal level (a level
similar to that by Ilv2 alone) upon an increase in the valine concentration. On the other hand,
the activities of AHAS reconstituted with the Ilv6 variants were slightly decreased even when
the valine concentration was increased up to 1.0 mM. These results supported the hypothesis
that amino acid substitutions within the valine-binding site remove valine feedback inhibition,
leading to intracellular valine accumulation (Fig. 6). The intracellular valine content (about 0.4% of dry cell weight) for the engineered strains (Fig. 5) corresponds to ca. 2 g valine per liter intracellular volume. This concentration is much higher than 1 mM valine tested in the in vitro assays (Fig. 6). The inhibition of AHAS activity from the wild-type Ilv6 reached the saturation at 0.4 mM valine (Fig. 6). We have not measured the valine inhibition at the concentration higher than 1.0 mM, since this range (0.4-1.0 mM) has been indicated to be the most suitable dose in order to see the difference between the wild-type and variants Ilv6.

It is noteworthy that Asn104 binds to valine upon dimer assembly, whereas Gly89 functions as a dimer interface between Ilv6 monomers (Fig. 4) (Phyre2: Kelley et al., 2015). When the binding site structure is altered, the interaction between the ligand and proteins may be perturbed. The 3-phosphoglycerate dehydrogenase (3-PGDH) is a good example; this enzyme also contains the ACT domain that is regulated through feedback inhibition by serine. In the serine-binding site, His404 acts as the major residue that binds to serine via the side-chains interaction between Asn406 and Asn424 (Grant et al., 1996; Chipman and Shaanan, 2001). This binding is thought to be based on polar-polar interaction, since histidine, serine and asparagine contain a polar side-chain. This assumption is complementary to a previous study in E. coli in which the amino acid replacement of AHAS IlvH at Gly14 and Asn29 resulted in an inability to bind with valine, based on the conserved residues of a 3-PGDH model (Grant et al., 1996; Mendel et al., 2001). However, the interaction between valine and its binding site in the AHAS regulatory subunit, Ilv6, is still unclear due to amino acid side-chains of the valine-binding site associating with polar and non-polar side chains. Meanwhile, the Asn86Ala, Gly89Asp and Asn104His variants displayed the DL-norvaline-resistant phenotype; the Val132Ile variant, which is also located at the ACT domain, was opposed. This variant was isolated as one of the DL-norvaline-resistant mutants from S. cerevisiae S288C (data not shown). This result suggests not only that the genes involved in the BCAA biosynthetic pathway can affect DL-norvaline
resistance by increasing valine productivity, but also that there are some upstream pathways
which play a role in DL-norvaline resistance in a valine-independent manner.

Amino acid substitutions at the carboxyl terminus of Ilv6, Ile255Ala/Arg and
Met276Ala/Asp, in S. cerevisiae did not have any effects on the DL-norvaline resistance or
intracellular valine, in contrast to the Leu131 and Val153 substitutions in the E. coli IlvH,
respectively. Both Leu131 and Val153 are located in the hydrophobic core of the ALS_ss_C
domain. Leu131 links between the α-4 helix and the β-sheet, and a mutation in this region can
affect the α-4 helix folding. Val153 is located between two monomers and is involved in the
inter-monomer interaction (Fig. 7A) (Kaplun et al., 2006). Homology modeling of the S.
cerevisiae Ilv6 (Fig. 7B), based on the E. coli IlvH, suggests that there are hydrogen bonds
between Ile255, Lys251 and Leu259 that are not directly involved in α-helix packing unlike the
corresponding position on the E. coli IlvH, Leu131, which bound to Thr127, Ser128, Phe134
and Leu153. This evidence supports the hypothesis that amino substitution at position 255 in
Ilv6 is not involved in the valine feedback inhibition in S. cerevisiae. Met275Ala/Asp also
appears not to be involved in valine feedback inhibition unlike Val153 of the E. coli IlvH.
However, there is no clear evidence to support this conjecture, since Met276 in the S. cerevisiae
Ilv6 is also located between two β-sheets and forms two hydrogen bonds between them, similar
to Val153 in E. coli (Fig. 7B). The ALS_ss_C domain of AHAS in S. cerevisiae might be
responsible for another ligand-binding, as described in a previous study, in which this domain
was responsible for the binding of MgATP (Pang and Duggleby, 2001). Taken together, this
evidence is consistent with our findings that the amino acid substitutions at Asn86, Gly89 and
Asn104 removed the valine-feedback inhibition (Fig. 6) and increased the intracellular valine
content by approximately 4-fold (Fig. 5) due to the conformational change in the valine-binding
site.
The results in Fig. 5 also show that the Ilv6 variants did not confer leucine and isoleucine accumulation. It is known that leucine and isoleucine biosynthesis originate from the same pathway as valine biosynthesis, although threonine is the substrate for isoleucine rather than pyruvate. KIV is the main precursor for both leucine and valine biosynthesis, and thus an increase in AHAS activity could certainly lead to intracellular accumulation of KIV. However, in our study, the accumulation of KIV or high AHAS activity did not increase the intracellular leucine and isoleucine. Leu4, α-isopropylmalate synthase I, is a rate-limiting step of leucine biosynthesis and subject to feedback inhibition by leucine (Kohlhaw, 2003). In the case of isoleucine, threonine deaminase (Ilv1) catalyzes the conversion of threonine to α-ketobutyrate, which is a starting point of isoleucine biosynthesis. It has been reported that this enzyme activity is inhibited by the presence of isoleucine (Berg et al., 2002). Therefore, neither more KIV...
availability nor higher AHAS activity can remove the feedback inhibition caused by leucine
and isoleucine, suggesting that, in *S. cerevisiae*, leucine and isoleucine feedback inhibitions are
regulated in an AHAS-independent manner. Unfortunately, intracellular leucine and isoleucine
have not been reported in a bacterial valine feedback inhibition-resistant AHAS strain, thus the
relationship between each BCAA feedback inhibition is still unknown. However, the leucine
level in *Arabidopsis thaliana* cells with valine feedback inhibition-resistant AHAS increased
by approximately 4-fold, whereas only a 3-fold increase was observed for valine (Chen et al.,
2010). In comparison, two repeats of the AHAS regulatory domain were observed in *A. thaliana*
(VAT1), although only a single domain is present in the yeast Ilv6. Moreover, the *A. thaliana*
AHAS activity is rather inhibited by all BCAAs, especially leucine (Lee and Duggleby, 2001),
suggesting that the regulatory mechanism of BCAA biosynthesis via AHAS is different in each
organism.

Comparing the valine biosynthesis between *E. coli* and *S. cerevisiae*, the primary substrate
for AHAS is pyruvate, although the *E. coli* AHAS III prefers α-ketobutyrate as the first substrate
(Gallop et al., 1990). Hence, in the presence of α-ketobutyrate, AHAS III catalyzes the synthesis
of isoleucine rather than valine (Pátek, 2007). Unlike that in bacteria, the AHAS activity in
yeast is partially inhibited by valine, and after the saturation point, additional increases in valine
concentration do not further inhibit the AHAS activity in yeast (Pang and Duggleby, 1999).
These results suggest that there is a different AHAS regulatory subunit in eukaryote cells.
Nevertheless, the regulation of valine biosynthesis via the AHAS regulatory subunit in *S.
cerevisiae* has not been fully clarified. The crystal structure of the yeast Ilv6 will be required to
resolve this issue.

5. Conclusions

An improvement of valine productivity would contribute not only to valine-related
industries but also to isobutanol production via the Ehrlich pathway (Avalos et al., 2013).

Researchers have attempted to increase intracellular valine levels in yeast by overexpressing the wild-type genes involved in valine biosynthesis. In this study, we introduced an alternative strategy by focusing on the feedback inhibition of AHAS activity by valine, which is a rate-limiting step in the valine biosynthesis of \textit{S. cerevisiae}. We successfully constructed several variants of the AHAS regulatory subunit Ilv6 (Asn86Ala, Gly89Asp and Asn104Ala) that were insensitive to the valine feedback inhibition compared with the wild-type protein by introducing amino acid substitutions based on previous results in IlvH, a similar protein in \textit{E. coli}. Moreover, we found that the intracellular valine content in yeast strains expressing these Ilv6 variants was approximately 4-fold higher than that of the wild-type strain. These results will contribute to the development of superior yeast strains for valine and isobutanol overproduction.

\textbf{Conflict of interest}

The authors declare there is no conflict of interest.

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Figure legends

Fig. 1. The metabolic pathways of from pyruvate to valine and isobuthanol in *S. cerevisiae*.
Protein names: Ilv2, the catalytic subunit of acetohydroxyacid synthase (AHAS); Ilv6, the regulatory subunit of acetohydroxyacid synthase (AHAS); Ilv5, acetohydroxyacid reductoisomerase; (AHAIR) Ilv3, dihydroxyacid dehydratase (DHAD); Bat1, mitochondrial BCAA aminotransferase (BCAT); Bat2, cytosolic BCAA aminotransferase (BCAT); Aro10, ketoacid (pyruvate) decarboxylase (KDC); Adh2, alcohol dehydrogenase (ADH).

Fig. 2. The amino acid sequence alignment of AHAS regulatory subunits, the *E. coli* IlvH and the *S. cerevisiae* Ilv6. (A) The schematic representation of the *E. coli* IlvH and the *S. cerevisiae* Ilv6 amino acid sequences. The ACT domain and the ALS_ss_C domain are orange and yellow, respectively. (B) The amino acid sequence alignment of the *E. coli* IlvH and the *S. cerevisiae* Ilv6. Residues N11, G14, N29, L131 and V153 in IlvH, where substitutions resulted in the resistance to feedback inhibition by valine (Kaplun et al., 2006), are indicated by red in orange and yellow box. The amino acid substitutions in Ilv6 are indicated by arrowhead below the sequences. The numbers are residue numbers. Identical and similar amino acids in the two proteins are indicated by the same residue and +, respectively. Dashes indicate the absence of corresponding amino acid residues at the positions.

Fig. 3. Growth phenotypes on DL-norvaline-containing medium of yeast cells expressing Ilv6. Cell suspensions with 10-fold serial dilutions were dropped (2.5 µL each) on SD agar medium, which was supplemented with 0.1% allantoin instead of ammonium sulfate as the sole nitrogen source, in the absence or presence of 10 mg/mL DL-norvaline, then incubated at 30 °C for 3 days.
Fig. 4. Homology modeling of the valine-binding site on the Ilv6 monomer. Based on the known *E. coli* IlvH molecular structure, the *S. cerevisiae* Ilv6 molecular structure was remodeled using Phyre2 and PyMOL. The positions of amino acid substitutions are indicated. Red, yellow and green indicate α-helix structure, β-sheet structure and a loop structure, respectively.

Fig. 5. Intracellular branched-chain amino acid contents. Yeast cells were grown in SD medium (pH 6.0) at 30°C and collected at log-phase (15 h after inoculation). White, light gray and dark gray bars represent intracellular valine, leucine and isoleucine contents, respectively. The values are the means and standard deviations of three independent experiments. Asterisks indicate statistically significant differences in comparison to the control (wild-type cells (BY4741) with the empty vector) (Tukey’s test, *p* < 0.05).

Fig. 6. Effects of valine on the AHAS specific activity. *In vitro* reconstituted complex of wild-type Ilv2 with wild-type Ilv6 or Asn86Ala, Gly89Asp or Asn104His variants of Ilv6 was used for the AHAS activity assay. Specific activity was expressed as enzyme units per mg of the catalytic subunit. The values are the means and standard deviations of three independent experiments. Asterisks indicate statistically significant differences in comparison to the variants of Ilv6 (Tukey’s test, *p* < 0.05).

Fig. 7. Amino acid substitution at the ALS_ss_C domain of yeast Ilv6. The ALS_ss_C domain of the *E. coli* IlvH (A) and the ALS_ss_C domain the of *S. cerevisiae* Ilv6 (B). Homology modeling was illustrated by Phyre2 and Pymol. All colors were given according to the secondary structure; red, yellow and green indicates a helix structure, a sheet structure and
a loop structure, respectively. The positions of amino acid substitutions were labeled as the stick with yellow dashes, which indicate hydrogen bonds.