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# High-level production of ornithine by expression of the feedback inhibition-insensitive *N*-acetyl glutamate kinase in the sake yeast *Saccharomyces cerevisiae*

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ABSTRACT

We previously reported that intracellular proline (Pro) confers tolerance to ethanol on the yeast *Saccharomyces cerevisiae*. In this study, to improve the ethanol productivity of sake, a traditional Japanese alcoholic beverage, we successfully isolated several Pro-accumulating mutants derived from diploid sake yeast of *S. cerevisiae* by a conventional mutagenesis. Interestingly, one of them (strain A902-4) produced more than 10-fold greater amounts of ornithine (Orn) and Pro compared to the parent strain (K901). Orn is a non-proteinogenic amino acid and a precursor of both arginine (Arg) and Pro. It has some physiological functions, such as amelioration of negative states such as lassitude and improvement of sleep quality. We also identified a homo-allelic mutation in the *ARG5,6* gene encoding the Thr340Ile variant *N*-acetylglutamate kinase (NAGK) in strain A902-4. The NAGK activity of the Thr340Ile variant was extremely insensitive to feedback inhibition by Arg, leading to intracellular Orn accumulation. This is the first report of the removal of feedback inhibition of NAGK activity in the industrial yeast, leading to high levels of intracellular Orn. Moreover, sake and sake cake brewed with strain A902-4 contained 4–5 times more Orn than those brewed with strain K901. The approach described here could be a practical method for the development of industrial yeast strains with overproduction of Orn.

## 1. Introduction

During the fermentation of sake, a traditional Japanese alcoholic beverage, the yeast *Saccharomyces cerevisiae* is exposed to high concentrations of ethanol which inhibits the growth, viability and fermentation of yeast cells (Kodama, 1993). Therefore, increased tolerance to ethanol in sake yeast strains could improve ethanol productivity and reduce fermentation time. Ethanol tolerance would also simplify the production process of bioethanol.

Intracellular proline (Pro) can be expected to alleviate the ethanol toxicity (Takagi et al., 2016). *S. cerevisiae* cells synthesize Pro from glutamate (Fig. 1). γ-Glutamyl kinase (GK) is the rate-limiting enzyme of Pro biosynthesis, and its activity is regulated allosterically by Pro (Sekine et al., 2007). *S. cerevisiae* has an alternative pathway for Pro synthesis from arginine (Arg) *via* ornithine (Orn) (Fig. 1). N -Acetyl glutamate kinase (NAGK) is one of the rate-limiting enzymesin Arg biosynthesis, and its activity is subject to feedback inhibition by Arg. Although the removal of feedback inhibition by amino acid substitution in NAGK has been reported in not only the laboratory *S. cerevisiae* strain (Pauwels et al., 2003) but also some bacteria, such as *Corynebacterium glutamicum* (Huang et al., 2015) and *C. crenatum* (Zhang et al., 2017), no such studies have been conducted in the industrial yeast strains including sake yeast.

To increase ethanol tolerance and productivity in sake yeast strains, we previously constructed strains that accumulate Pro by replacing the wild-type *PRO1* allele with the *PRO1*$^{D154N}$ or *PRO1*$^{I150T}$ allele (Takagi et al., 2005, 2007). The resultant strains were more tolerant to ethanol than the wild-type strain (Takagi et al., 2005). The Pro-accumulating strains also produced higher amounts of ethanol than the parent strain (Takagi et al., 2007). However, the above-mentioned yeasts are not yet acceptable to consumers as they are considered to be genetically modified, even though a self-cloning yeast contains no foreign genes or DNA sequences except for yeast DNA (Takagi et al., 2007).

In general, microorganisms that overproduce various amino acids

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have been obtained by isolating mutants resistant to the toxic analogues of those amino acids. In the present study, we isolated Pro-accumulating mutants derived from diploid sake yeast strains that were resistant to the Pro toxic analog azetidine-2-carboxylate (AZC) by a conventional mutagenesis. Interestingly, two of the mutants accumulated not only Pro but also Orn in the cell. Sake brewed with these mutants also contained more Pro and Orn than sake brewed with the parent strain. Orn is an important precursor of Arg, and is biosynthesized from glutamate in the mitochondria and then transported to the cytoplasm. It is a non-proteinogenic amino acid that functions in the urea cycle. The urea cycle detoxifies ammonia generated by the degradation of amino acids in protein to urea. High amounts of Orn are contained in various organisms and foods (Manzi et al., 1999; Uchisawa et al., 2004; Niro et al., 2017). Orn has been reported to improve various negative physical or emotional states in humans, including fatigue and lassitude (Kokubo et al., 2013). Thus, Orn-rich sake would be expected to relieve feelings of fatigue after drinking.

However, the molecular mechanisms involved in both higher Pro and Orn production in the AZC-resistant mutants have not yet been clarified. Here, we identified and analyzed a novel mutation in the *ARG5,6* gene encoding the Thr340Ile variant of NAGK, which increases intracellular Pro and Orn levels.

## 2. Materials and methods

### 2.1. Strains and culture media

In this study, we used *S. cerevisiae* strain BY4741 (*MAT*a *his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0) derived from strain S288C, and a diploid sake strain Kyokai No. 901 (K901), which is widely used in Japanese sake breweries. Strain BY4741 *arg5,6*Δ (BY4741*arg5,6::kanMX6*), which was provided by EUROSCARF (Oberursel, Germany), was used for the expression the wild-type and mutant *ARG5,6* genes.

The media used for growth of *S. cerevisiae* were a nutrient-rich YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose), 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), SD medium containing 0.04% L-leucine, 0.008% L-histidine, L-methionine (SD + Leu + His + Met), and a synthetic complete medium SC-ura (0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA), and 0.2% drop-out mix lacking uracil). The dropout mix contains 0.002% adenine, 0.0008% *p*-aminobenzoic acid, 0.04% L-leucine, 0.008% inositol, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine hydrochloride, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine. AZC was supplemented at a concentration of 1 mg/mL for the screening of proline-accumulating cells.

An *E. coli* strain DH5α (*F-, ϕ80dlacZ*Δ*M15,* Δ*(lacZYA-argF)U169, deoR, recA1, endA1, hdR17* ($r_k^-$, $m_k^+$)*, phoA, supE44,* $\lambda^-$*, thi-1, gyrA96, relA1*) was used for plasmid propagation and the bacterial transformation was carried out by the method for high efficiency transformation (Inoue et al., 1990). An *E. coli* strain BL21 (DE3) [$F^-$ *omp*T *hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)] was used for expression and purification of the recombinant NAGK proteins. The *E. coli* recombinant strains were cultured in Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) containing 100 μg/mL of ampicillin. If necessary, 2% agar was added to solidify the medium.

### 2.2. Isolation of Pro-analog-resistance mutants

The diploid sake strain K901 was randomly mutagenized by treatment with 4% ethyl methanesulfonate in 100 mM phosphate buffer (pH 7.0) at 30 °C for 1 h (Rose and Broach, 1991). Mutagenized cells were washed with 10% sodium thiosulfate and then twice with sterilized water. Approximately 2–4 × $10^6$ cells were spread onto SD agar plates containing 1 mg/ml AZC. After incubation at 30 °C for 2–3 days, the resulting colonies were collected and tested for amino acid production.

### 2.3. Measurement of intracellular amino acids content

Yeast cells were cultured in 5 ml of SD or SD + Leu + His + Met. After overnight cultivation at 30 °C, the cultured cells were inoculated into 5 ml of the above medium at a final optical density 600 nm ($OD_{600}$) of 0.25. After cultivation for 48 h at 30 °C with rotary shaking, cells ($OD_{600}$ = 10) were washed twice and intracellular amino acids were extracted

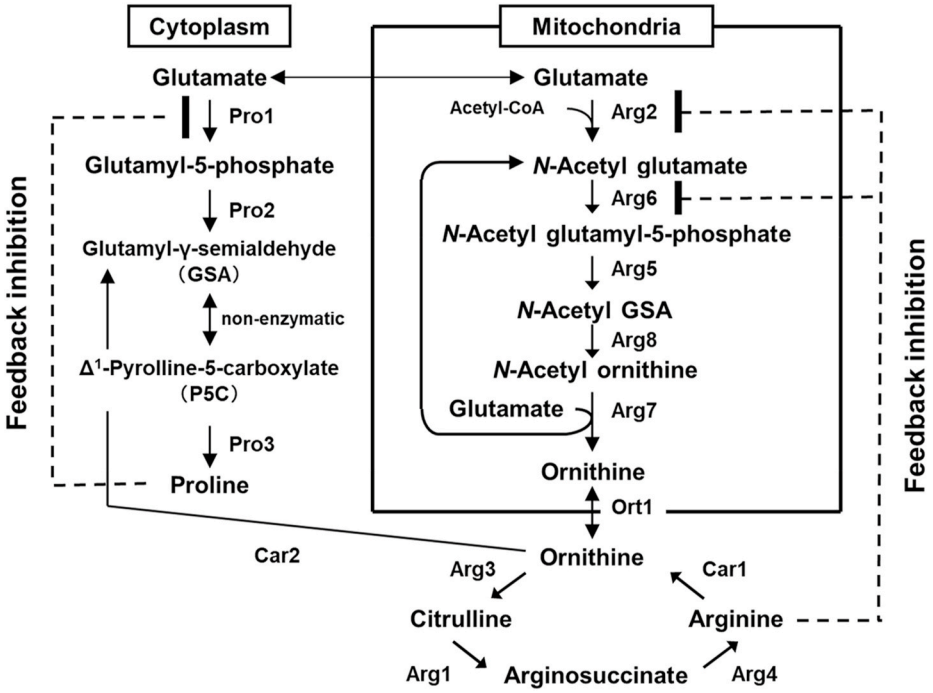


**Fig. 1. Metabolic pathways of Pro, Orn and Arg in *S. cerevisiae*.** Protein names: Pro1, γ-glutamyl kinase; Pro2, γ-glutamyl phosphate reductase; Pro3, P5C reductase; Arg2, *N*-acetyl-glutamate synthase; Arg6, *N*-acetyl-glutamate kinase; Arg5, *N*-acetyl-glutamyl-5-phospate reductase; Arg8, *N*-acetyl-Orn aminotransferase; Arg7, *N*-acetyl-Orn acetyltransferase; Ort1, Orn transporter; Arg3, Orn carbamoyltransferase; Arg1, arginosuccinate synthetase; Arg4, arginosuccinate lyase; Car1, arginase; Car2, Orn aminotransferase.

by boiling water for 20 min. After centrifugation (5 min at 15,000×*g*), each supernatant was subsequently quantified with an amino acid analyzer with ion-exchange chromatography and post-column ninhydrin derivatization (JLC-500/V, JEOL, Tokyo, Japan), or with an UF-Amino Station (Shimadzu, Kyoto, Japan) with pre-column derivatization using 3-aminopyridyl-N-hydroxysuccinimidyl carbamate (Wako Pure Chemical, Osaka, Japan). The content of each amino acid was expressed as a percentage of dry weight.

### 2.4. Laboratory-scale brewing tests with sake yeast strains

For laboratory-scale sake brewing tests, we used strain K901 and proline-accumulating mutants. A single-step sake mash was prepared by mixing 36.4 g pregelatinized rice, 9.6 g dried koji (rice cultivated with *Aspergillus oryzae*), 50 μl 90% lactic acid, and 87 ml water containing yeast cells at a final $OD_{600}$ of 0.6 and was then incubated at 15 °C for 28 days without shaking. Fermentation progression was continuously monitored by measuring the weight loss due to $CO_2$ gas production. Sake mash was centrifuged, and the supernatant was obtained as fresh sake, then the residue was obtained as sake cake. For determination of amino acids concentration, the samples were prepared as follows. Sake samples were diluted by five-fold with de-ionized water and then filtered through a 0.45-μm membrane filter. Amino acids in 5 g of sake cake were extracted by boiling water for 30 min. After centrifugation (5 min at 15,000×*g*), the supernatant was collected and made up to 25 ml with de-ionized water and then the samples were filtered through a 0.45-μm membrane filter. Amino acid contents in sake and sake cake was quantified with an amino acid analyzer or an UF-Amino Station.

### 2.5. Whole genome sequencing analysis

The extracted genome DNA from the AZC-resistant strains (A901–8 and A902-4) and the parent strain K901 underwent quantification by Qubit (Thermo Fisher Scientific, Waltham, MA, USA). Next generation sequencing library was constructed for each genome using the Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The genome libraries were sequenced using MiSeq (Illumina) with MiSeq Reagent Kit v2 or v3 (Illumina). Sequencing data processing of A901–8, A902–4 and K901, as well as sequencing data from the Sequence Read Archive (SRA) was performed with CLC Genomics Workbench v 10.1.1 (Quiagen, Hilden, Germany). This included trimming, mapping and variants calling against the reference genome of *S. cerevisiae* S288c (GCA_000146045). Reads bases not matching in the alignment were scored as variants. The coverage table files and the variants table files were exported from Genomics Workbench and retained for further analysis. These files were converted into a fasta file of synthetic sequences with custom scripts. These scripts generate the sequences of homozygous SNPs from the data of coverage and variants.

A Venn diagrams was created using the numbers of genes that have mutations with the amino acid substitutions compared with the *S. cerevisiae* laboratory strain S288C between two strains from strains A901–8, A902–4 and K901.

### 2.6. Construction of expression plasmids for the ARG5,6 genes

For the construction of yeast centromere plasmid (Ycp) pRS416-ARG5,6 expressing the wild-type *ARG5,6* gene, the coding region of the *ARG5,6* gene containing 1000 bp both upstream and downstream sequences was amplified from the genomic DNA of strain BY4741 by high-fidelity PCR using KOD Plus DNA polymerase using primers ARG5,6 Fw (HindIII) and ARG5,6 Rv (BamHI) (Supplementary Table S1) and was cloned into the *Hin*dIII-*Bam*HI site of pRS416 by In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) to construct pRS416-ARG5,6. In-Fusion products were then digested with *Dpn*I before introduction into *E. coli* DH5α cells. Plasmids pRS416-ARG5,6 (T340I) expressing the mutated *ARG5,6* genes were constructed from pRS416-ARG5,6 using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with primers ARG5,6 (T340I) Fw and ARG5,6 (T340I) Rv (Supplementary Table S1). PCR products were then digested with *Dpn*I before introduction into *E. coli* DH5α cells. Plasmids pRS416-ARG5,6 and pRS416-ARG5,6 (T340I) were further confirmed by DNA sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA), respectively. Strain BY4741 *arg5,6*Δ was transformed with pRS416 harboring the wild-type *ARG5,6* or the mutant *ARG5,6*$^{T340I}$ gene by the lithium acetate method with slight modification. Yeast transformants were selected on SC-ura agar plates.

To express and purify the recombinant NAGK from *E. coli*, we constructed expression plasmids as described below. The *ARG5,6* gene encodes a polyprotein precursor with the amino-terminal NAGK and the carboxyl-terminal *N*-acetyl glutamyl phosphate reductase (Boonchird et al., 1991) The polyprotein precursor is cleaved into the two enzymes in the mitochondria (Abadjieva et al., 2001), and then deletion of amino-terminal mitochondrial targeting signal sequence (residue 1–57 (Boonchird et al., 1991)) results in yeast mature NAGK by proteolytic processing. To produce NAGK, which lacks the mitochondria targeting sequence, the DNA sequence encoding enzyme residue 58–513 was amplified from pRS416-ARG5,6 or pRS416-ARG5,6 (T340I) by high-fidelity PCR using KOD Plus DNA polymerase using primers pQE-2 NdHi Fw (NdeI) and pQE-2 NdHi Rv (HindIII) (Supplementary Table S1) and was cloned into the *Nde*I-*Hin*dIII site of pQE-2 (Quiagen) by In-Fusion HD Cloning Kit (Takara Bio) to construct pQE-2-NAGK-WT or pQE-2-NAGK-T340I. In-Fusion products were then digested with *Dpn*I before introduction into Zymo 5α cells (Zymo Research, Irvine, CA, USA). Plasmids pQE-2-NAGK-WT and pQE-2-NAGK-T340I were further confirmed by DNA sequencing, respectively. An *E. coli* strain BL21 (DE3) was transformed with pQE-2-NAGK-WT or pQE-2-NAGK-T340I.

### 2.7. Expression and purification of recombinant NAGKs

*E. coli* transformant cells carrying pQE-2-NAGK-WT or pQE-2-NAGK-T340I were pre-cultured at 37 °C for overnight in LB medium containing 100 μg/mL of ampicillin. The main culture was performed by inoculating cells into a 300 mL flask at the initial $OD_{600}$ of 0.05 and incubating at 37 °C until $OD_{600}$ was reached to 0.6–0.8. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and cultured at 18 °C 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 °C for 10 min at 3000 rpm. The cell pellet was washed with ice-cold sonication buffer (10 mM $MgCl_2$, 0.25 M NaCl, 20 mM Tris-HCl, pH 7.5), and the intact cells were stored at −80 °C if they were not used immediately.

For purification of recombinant NAGKs, cell pellets were suspended in ice-cold sonication buffer. Cell mixture was subjected to sonication (output = 7, 10 s/on ice 30 s for 4 cycles) by Ultrasonic generator US-150T (Nihonseiki, Tokyo, Japan) and was subsequently centrifuged at 15,000 rpm for 20 min at 4 °C, and then filtrated by 0.45 μm diameter filter. Crude protein was loaded by gravity on Ni Sepharose 6 Fast Flow resin (GE Healthcare, Chicago, IL, USA), equilibrated with sonication buffer. The resin was washed sonication buffer with 20 mM imidazole and then with 70 mM imidazole-containing sonication buffer. Purified proteins were eluted by 500 mM imidazole-containing buffer. Proteins were quantified using Bio-Rad Protein Assay (Hercules, CA, USA) and subjected to SDS-polyacrylamide gel electrophoresis.

### 2.8. Assay of NAGK activity

NAGK activity was determined by coupling the production of ADP with the lactate dehydrogenase (LDH)-dependent oxidation of reduced NADH in the presence of phosphoenol pyruvate (PEP) and pyruvate kinase (PK) using a Synergy HTX microplate reader (Bio-Tek

Instruments, Winooski, VT, USA). In this PK/LDH-coupled enzyme system, ADP produced by phosphorylation of *N*-acetyl glutamate (NAG) is phosphorylated by phosphoenol pyruvate, catalyzed by PK. The pyruvate in this reaction is reduced to lactate by NADH, catalyzed by LDH. Therefore, NAGK activity is measured by monitoring decrease of NADH. In standard assay conditions, NAGK reaction mixture contained 25 mM NAG, 5 mM ATP, 10 mM MgCl2, 1 mM PEP, 20 U PK, 30 U LDH enzymes from rabbit muscle (Sigma-Aldrich, St. Louis, MO, USA), 0.25 mM NADH, 100 mM HEPES (pH 7.5), and 5 μg of NAGK in a final volume of 300 μL. The NAGK reaction mixture was pre-warmed at 30 °C for 1 min without NAG, and then NAG was added to start the NAGK reaction. The rate of the decrease in absorption at 340 nm was measured consecutively. NAGK activity was calculated from the initial linear rate with the extinction coefficient ($\varepsilon_{340\ nm} = 6.22\ mM^{-1}cm^{-1}$) of NADH. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of $NAD^+$ per min. To determine the inhibition profiles, NAGK activity was measured by adding Arg (final concentrations of 0, 0.2, 0.4, 0.6, 1, 2, 5 or 10 mM), Orn (10 mM) or citrulline (10 mM) in the standard assay conditions.

### 2.9. Structural analysis

To analyze the effect of amino acid substitution in NAGK, we conducted structural comparison of NAGK from various organisms using the structure of NAGK bound to arginine from *S. cerevisiae* (PDB ID code: 3ZZH) as the template structure. Figures of NAGK structures were drawn using PyMOL (http://www.pymol.org).

## 3. Results

### 3.1. Isolation of sake yeast mutants with Pro and Orn accumulation from AZC-resistant mutants

Intracellular Pro, which is accumulated in AZC-resistant mutants, could play a role in reducing ethanol stress (Takagi et al., 2005). AZC is a toxic analog of Pro in both laboratory and sake yeasts (Kimura et al., 2002; Ohashi et al., 2019). In fact, 1 mg/mL of AZC strongly inhibited the growth of the diploid sake yeast strain K901. To obtain Pro-accumulating sake yeast mutants with enhanced ethanol tolerance, we attempted to isolate AZC-resistant mutants from the parent strain K901. When yeast cells treated with ethyl methanesulfonate were directly plated on SD agar medium containing AZC, approximately 20 AZC-resistant colonies were totally obtained from two independent experiments.

We next measured intracellular amino acids content in AZC-resistant mutants. Among the AZC-resistant mutants, three (A901–8, A902–4 and A902-6) had higher levels of intracellular Pro than the parent strain K901, with differences as high as 9.7-, 10.4- and 6.4-fold, respectively (Fig. 2A). Interestingly, we found that strains A902–4 and A902-6 accumulated not only Pro but also Orn (Fig. 2B). Strains A902–4 and A902-6 showed 13.3-fold and 7.3-fold increases, respectively, in Orn level compared to strain K901. However, intracellular Arg, which is biosynthesized from Orn, in strains A902–4 and A902-6 was not notably increased compared to strain K901 (Fig. 2C). Based on these results, strains A901-8 with Pro accumulation and A902-4 with Pro and Orn accumulation were selected for further analysis.

### 3.2. Whole genome sequence analysis of Pro- and Orn-accumulating sake yeast mutants

We then conducted next-generation sequencing for the whole genome of strains A901–8, A902–4 and K901 in order to identify the mutations that conferred Pro and Orn accumulation on yeast cells. As shown in Fig. S1, all strains had the gene mutations of approximately 3500 in common. However, we identified 256 and 248 gene mutations with amino acid substitutions in the open reading frame in strains A901–8 and A902-4, respectively, compared to strain K901. Among them, we first focused on the genes related to Pro and Orn biosynthesis, such as *PRO1*, *PRO2*, *PRO3 ARG1*, *ARG2*, *ARG3*, *ARG4*, *ARG5,6*, *ARG7*, *ARG8*, *ORT1*, *CAR1*, and *CAR2* (Fig. 1).

In strain A901-8, the *PRO1* gene sequence contained a homozygous mutation, i.e., a single base change at position 739, which led to one amino acid replacement of Pro to Ser at position 247 in GK. This mutation found in baker's yeast resulted in AZC resistance of yeast cells and desensitization to Pro feedback inhibition of GK, leading to intracellular Pro accumulation (Tsolmonbaatar et al., 2016). In addition, the *CAR2* gene sequence contained a heterozygous mutation, i.e., a single base change at position 245, which led to one amino replacement of Ala to Val.

Interestingly, we found that the *ARG5,6* gene sequence in strain A902-4 included a homozygous mutation, which was a single base change at position 1,019, leading to one amino acid change of Thr to Ile at position 340 in NAGK. On the other hand, strains A902–4 and A901-8 had no mutations in the *PRO1* gene leading to Pro overproduction or the *ARG5,6* gene leading to Orn overproduction, respectively.

### 3.3. Effect of the ARG5,6 mutation on intracellular amino acids content

To analyze the *ARG5,6* gene mutation found in strain A902-4, we constructed the expression plasmids for the wild-type and mutated *ARG5,6* (*ARG5,6*$^{T340I}$) genes. Strain BY4741 *arg5,6*Δ cells expressing the wild-type *ARG5,6* and the mutant *ARG5,6*$^{T340I}$ genes were cultivated in SD + Leu + His + Met liquid medium, and the cellular amino acid levels were determined (Fig. 3). We found that the Pro and Orn content in cells expressing the *ARG5,6*$^{T340I}$ gene were 4.1-fold and 7.4-fold higher, respectively, than those of cells expressing the *ARG5,6* gene (Fig. 3A and

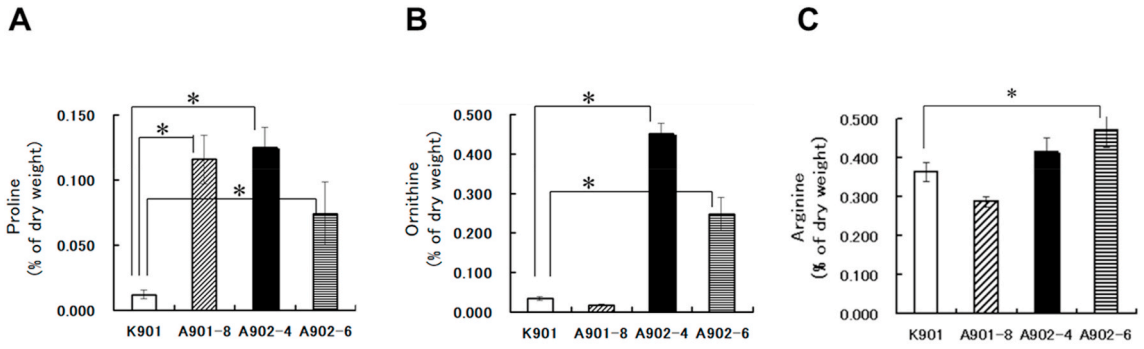


**Fig. 2. Intracellular (A) Pro, (B) Orn and (C) Arg content.** Sake yeast cells were cultured in SD liquid medium at 30 °C for 48 h. The values were means and standard deviation of results from three independent experiments. Asterisks indicate statistically significant differences between the parent strain K901 and AZC-resistant mutant strains A901–8, A902–4, A902–6 and K901, respectively (Student's *t*-test, $p < 0.05$).

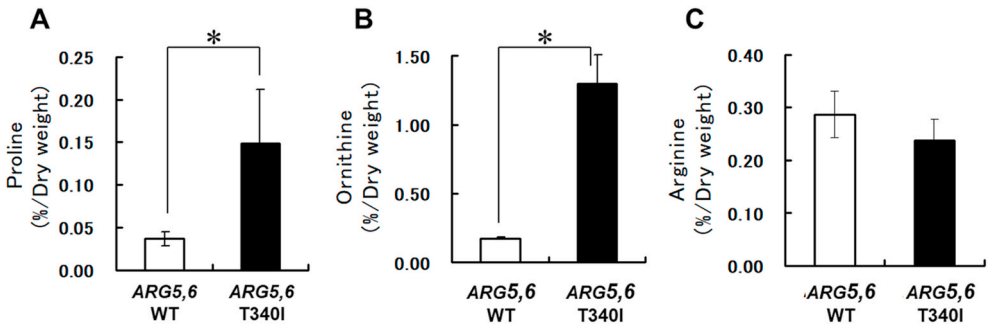


**Fig. 3. Intracellular (A) Pro, (B) Orn and (C) Arg content.** BY4741 *arg5,6*Δ cells expressing the wild-type *ARG5,6* (WT) and the mutant *ARG5,6*$^{T340I}$ (T340I) genes were cultured in SD + Leu + His + Met liquid medium at 30 °C for 48 h. The values were means and standard deviation of results from three independent experiments. Asterisks indicate statistically significant differences between WT and T340I (Student's *t*-test, $p < 0.05$).

B). However, no significant effect of the *ARG5,6* mutation on Arg production was observed in yeast cells expressing the *ARG5,6*$^{T340I}$ gene (Fig. 3C).

### 3.4. Effect of amino acid substitution of NAGK on NAGK activity

The polyprotein precursor encoded by the *ARG5,6* gene is likely cleaved into the two enzymes NAGK and *N*-acetyl glutamyl phosphate reductase between residues 510 and 540 (Abadjieva et al., 2001). Therefore, the amino acid substitution at position 340 might affect the enzymatic activity of NAGK. NAGK activity is inhibited in the presence of Arg (Pauwels et al., 2003). Since the mutant *ARG5,6*$^{T340I}$ greatly elevated the intracellular Orn content (Fig. 3B), we hypothesized that the amino acid substitution at position 340 induced some conformational change of the Arg-binding site, leading to reduction of Arg-binding affinity. In order to examine this hypothesis, we expressed, purified the recombinant NAGK-WT and NAGK-T340I proteins tagged with 6 × His at the amino terminus from *E. coli* BL21 (DE3) cells (Fig. S2) and measured the NAGK activity of purified NAGK-WT and NAGK-T340I (Fig. 4).

In the absence of Arg, NAGK-T340I activity (1.57 ± 0.10 U/mg) was slightly lower than that of the wild-type NAGK (1.73 ± 0.06 U/mg). However, NAGK-WT activity drastically decreased by addition of 0.2 mM Arg, and consequently decreased when Arg concentration was increased, indicating that the NAGK activity is subject to feedback inhibition by Arg. NAGK-WT activity was almost lost in the presence of 1.0 mM Arg (Fig. 4), but was not inhibited by 10 mM Orn (1.63 ± 0.06 U/mg) or 10 mM citrulline (1.60 ± 0.00 U/mg). By contrast, NAGK-T340I activity was largely insensitive to feedback inhibition at Arg concentration up to 1 mM. It is noteworthy that, even in the presence of 10 mM Arg, NAGK-T340I exhibited approximately 80% of the activity observed in the absence of Arg (data not shown).

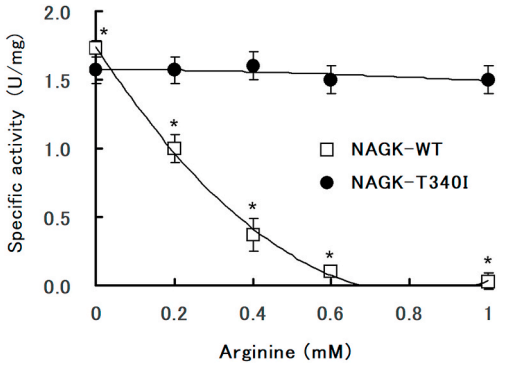


**Fig. 4. Effects of Arg on the NAGK specific activity.** The recombinant wild-type (NAGK-WT) and Thr340Ile variant (NAGK-T340I) NAGKs were used for the enzyme activity assay. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of $NAD^+$ per min. The values are the means and standard deviations of three independent experiments. Asterisks indicate statistically significant differences in comparison to NAGK-T340I (Student *t*-test, $p < 0.05$).

### 3.5. Sake fermentation test for Pro- and Orn-accumulating sake yeast mutant

Finally, we brewed laboratory-scale sake with the parent strain K901 and the Pro- and Orn-accumulating strain A902-4 and analyzed their fermentation profiles. After 28 days of fermentation, there was no significant difference in total $CO_2$ emissions between strain A902-4 and strain K901, and strain A902-4 produced a similar amount of ethanol (20.4 ± 0.6%) to strain K901 (19.5 ± 0.1%). As we expected, sake and sake cake brewed with strain A902-4 contained 5.0 times and 4.2 times more Orn, respectively (Fig. 5A and C), than those brewed with strain K901. In addition, Pro content was significantly increased (30–40%) in sake and sake cake brewed with strain A902-4 compared to strain K901 (Fig. 5B and D).

## 4. Discussion

GK is the key enzyme regulating Pro biosynthesis in *S. cerevisiae*, and GK activity is allosterically subjected to feedback inhibition by Pro (Sekine et al., 2007). However, we previously reported that GK variants such as Ile150Thr (Sekine et al., 2007) and Pro247Ser (Tsolmonbaatar et al., 2016) were insensitive to feedback inhibition by Pro. In this study, the Pro-accumulating strain A901-8 (Fig. 2A) had a homo-allelic mutation in the *PRO1* gene, which encodes the Pro247Ser variant GK. Therefore, it strongly suggested that the Pro-to-Ser substitution at position 247 in GK reduces sensitivity to Pro feedback inhibition, leading to high levels of Pro in yeast cells. In strain A901-8, there was a hetero-allelic mutation in the *CAR2* gene, which encodes the Ala82Val Orn transaminase variant, but it is unclear whether this mutation causes Pro overproduction. On the other hand, strain A902-4, which accumulates both Pro and Orn (Fig. 2B), has a homozygous mutation in the *ARG5,6* gene, leading to the Thr340Ile substitution in NAGK. Expression of the *ARG5,6*$^{T340I}$ gene in strain BY4741 *arg5,6*Δ markedly increased the intracellular Pro and Orn content compared to those of strain BY4741 expressing the wild-type *ARG5,6* gene (Fig. 3A and B). Although there might be the other genomic mutations which contribute to the Orn overproduction in strain A902-4, these results indicate that the amino acid replacement of Thr to Ile at position 340 in NAGK confers Pro and Orn accumulation in yeast cells.

NAGK activity is subject to Arg feedback inhibition in most microorganisms, including *S. cerevisiae* (Pauwels et al., 2003), *Pseudomonas aeruginosa* (Fernandez-Murga and Rubio, 2008), *Thermotoga maritima* (Ramón-Maiques et al., 2006), *C. glutamicum* (Sakanyan et al., 1996) and *Synechococcus elongatus* (Maheswaran et al., 2004). However, the

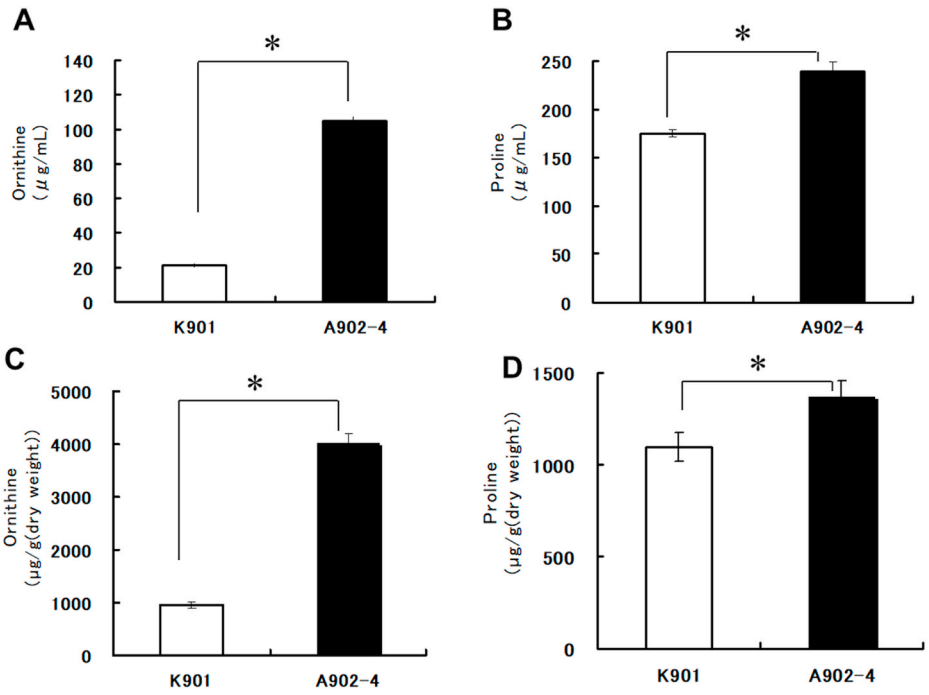


**Fig. 5. Orn and Pro content in (A, B) sake and (C, D) sake cake.** Sake and sake cake was brewed with the parent strain K901 and the Pro- and Orn-accumulating strain A902-4. The values were means and standard deviation of results from three independent experiments. Asterisks indicate statistically significant differences between strains K901 and A902-4 (Student's *t*-test, $p < 0.05$).

NAGKs of *E. coli* (Cunin et al., 1986) and *Thermus thermophilus* (Sundaresan et al., 2012) are not inhibited by Arg. In addition, the NAGK variant (Phe99Leu) of the genetically modified *S. cerevisiae* is resistant to feedback inhibition by Arg (Pauwels et al., 2003). The activity of the Phe99leu variant was only one half of that of the wild-type NAGK in the absence of Arg and decreased up to 50% in the presence of 10 mM Arg compared with that in the absence of arginine. In contrast, the Thr340Ile variant NAGK caused desensitization to Arg feedback inhibition compared to the wild-type enzyme, although its specific activity was slightly lower than that of the wild-type NAGK (Fig. 4). The three-dimensional structure of NAGK from *S. cerevisiae* (ScNAGK) has been previously determined (de Cima et al., 2012). It was observed that Arg interacts with Lys265, Ser285, Lys336, Glu337, Thr340 and Gly345 in ScNAGK (Fig. 6A). The multiple alignments indicated that Lys265 and Thr340 were conserved only in NAGKs that have been reported to be sensitive to feedback inhibition by Arg (Fig. 6B), suggesting that these residues are involved in Arg recognition. Thr340 binds to Arg *via* its carbonyl oxygen in the main-chain, whereas it also interacts with Lys336 through its hydroxyl group in the side-chain (Fig. 6A). Because Thr340 was conserved among Arg-sensitive NAGKs as Thr or Ser, the side-chain hydroxyl group at position 340 could be important for Arg binding. Comparison of local folding of various NAGKs revealed that local conformation around Thr340 forming the Arg-binding cavity is strictly conserved among NAGKs, which have been reported to be sensitive to feedback inhibition by Arg (Beez et al., 2009; Huang et al., 2015; Maheswaran et al., 2004; Ramón-Maiques et al., 2006) (Fig. 6C). On the other hand, Arg-insensitive NAGKs exhibited the different local folding in this region (Gil-Ortiz et al., 2010; Sundaresan et al., 2012). The difference of this local structure was one of the most important mechanisms for explaining the Arg sensitivity of NAGKs. Thr340 seems to be critical for stabilizing this local conformation through the intramolecular interaction with Lys336. Therefore, we concluded that the substitution of Thr340 to Ile disrupts the local structure *via* the intramolecular interaction, leading to desensitization of NAGK to Arg.

Orn is generated in two different metabolic pathways. In the linear pathway, it is synthesized through the hydrolysis of *N*-acetyl Orn in some bacteria and archea, such as *E. coli* (Cunin et al., 1986) and *Sulfolobus solfataricus* (Van de Casteele et al., 1990). In the cyclic pathway, transfer of the acetyl group of *N*-acetyl Orn to glutamate generates Orn, thereby regenerating *N*-acetyl glutamate not only in prokaryotes, such as *Bacillus stearothermophilus* (Sakanyan et al., 1992) and *Streptomyces coelicolor* (Hindle et al., 1994) but also in eukaryotes, such as *Candida utilis* (Middelhoven, 1963), *Neurospora crassa* (Davis, 1986) and *S. cerevisiae* (Pauwels et al., 2003). *N*-Acetyl glutamate synthase (NAGS), which is encoded by the *ARG2* gene and catalyzes the N-acetylation of glutamate to produce *N*-acetyl glutamate, is also subject to feedback inhibition by Arg in *S. cerevisiae* (Pauwels et al., 2003) (Fig. 1). Therefore, NAGS in strain A902-4 of the Pro- and Orn-accumulating mutant is also likely inhibited by Arg. Thus, the level of intracellular *N*-acetyl glutamate is strongly regulated by NAGS activity, which is subject to Arg feedback inhibition.

The T340I variant NAGK in strain A902-4 is not inhibited by Arg, however, and the following enzyme reaction proceeds. *N*-Acetyl glutamate is regenerated with the production of Orn by transferring the acetyl group from *N*-acetyl Orn to glutamate in the cyclic pathway, resulting in overproduction of Orn in strain A902-4. On the other hand, we were not able to find any mutations associated with Pro accumulation in the *PRO1* gene in strain A902-4, suggesting that GK activity may be inhibited in the presence of Pro. However, based on the fact that *S. cerevisiae* cells can synthesize Pro from Orn under catalysis by Orn transaminase (Kaino et al., 2012), strain A902-4 likely synthesizes Pro from excess Orn.

Previous studies have reported that removal of Arg feedback inhibition of NAGK increased the production of Arg in *C. glutamicum* cells that overexpress the mutated genes (Huang et al., 2015; Ikeda et al., 2009). In this study, however, expression of the Thr340Ile variant NAGK, which is insensitive to feedback inhibition by Arg, did not markedly elevate the Arg level in yeast cells (Fig. 3C). The activity of Orn carbamoyltransferase (OTC) encoded by the *ARG3* gene is decreased in the presence of Arg in *S. cerevisiae* (Messenguy and Dubois, 1983). Therefore, expression of the Thr340Ile variant NAGK might have only a minimal effect on the intracellular Arg content due to the inhibition of OTC by Arg, but if so, further studies will be needed to clarify

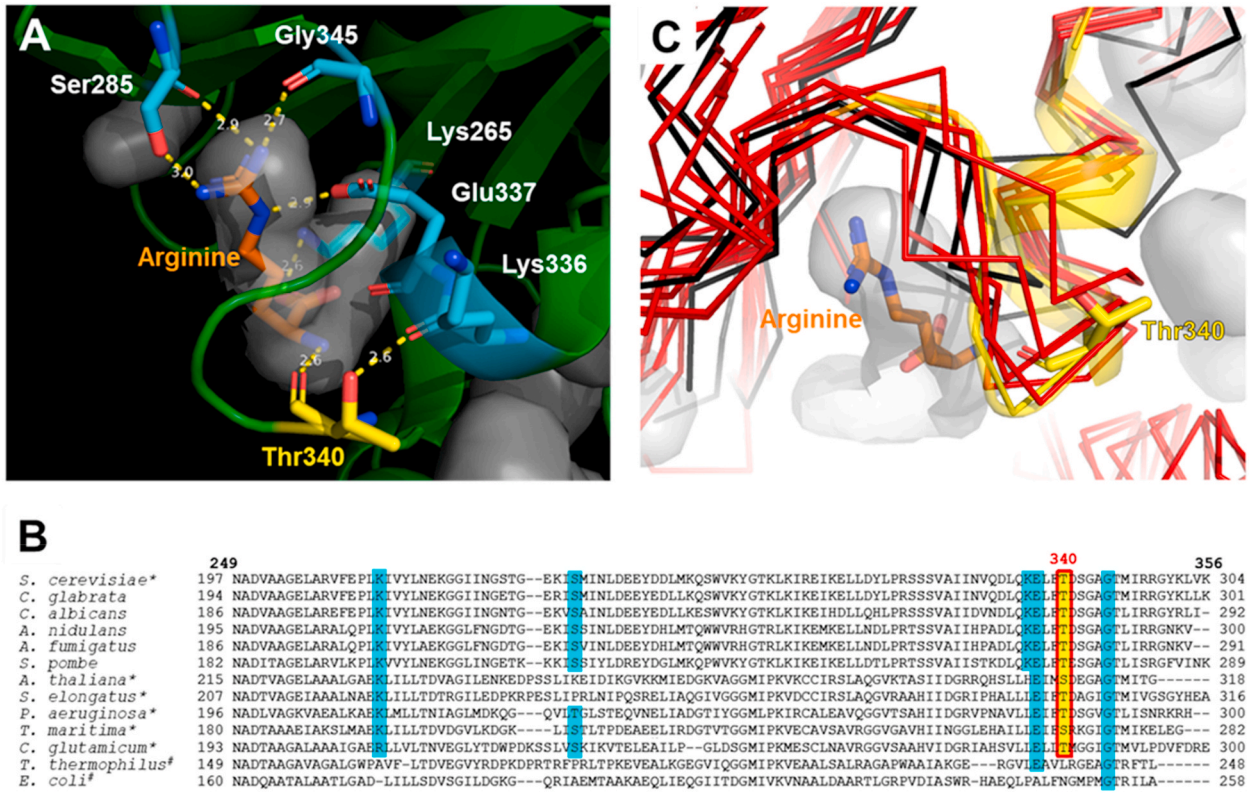


**Fig. 6. Structural comparison of NAGK from various organisms.** (A) Structure of the Arg-binding site in the *S. cerevisiae* NAGK (ScNAGK) (PDB ID code: 3ZZH). A whole protein structure was shown by a cartoon model. The residues which interact with Arg were shown in a stick model with cyan color, except for Thr340, which was shown in yellow color. The expected hydrogen bonds were shown in yellow dots with their distances in Å. Arg was shown in a stick model with orange color. The cavities were also shown. (B) Multiple alignment of NAGKs from various organisms by ClustalW. Numbering of residues is in ScNAGK. Among the residues shown in (A), conserved residues were highlighted in cyan or yellow boxes. * or # indicates an organism with Arg-sensitive or -insensitive NAGK, respectively. (C) Multiple superimposition of NAGK 3D structures from various organisms around the Arg-binding site in ScNAGK. Backbones of whole protein structures were shown in a ribbon model. The red ribbons show NAGK from *S. cerevisiae* (3ZZH), *Thermotoga maritima* (2BTY), *Pseudomonas aeruginosa* (2BUF), *Synechococcus elongatus* (2V5H) or *Arabidopsis thaliana* (4USJ), which were reported to be sensitive to Arg feedback inhibition (Beez et al., 2009; de Cima et al., 2012; Huang et al., 2015; Maheswaran et al., 2004; Ramón-Maiques et al., 2006). The black ribbons show NAGK from *Escherichia coli* (1GS5) or *Thermus thermophilus* (3U6U), both of which are suggested not to be inhibited by Arg (Gil-Ortiz et al., 2010; Sundaresan et al., 2012). Arg and cavities belonging to ScNAGK were also shown. Thr340 and its local region in ScNAGK were shown by a stick or cartoon model in yellow color, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the molecular mechanism.

Orn overproducers have been developed using metabolic engineering in genetically modified microorganisms, such as *C. glutamicum* (Kim et al., 2015), *C. crenatum* (Jiang et al., 2013) and *S. cerevisiae* (Qin et al., 2015). These microorganisms could not be directly applied in food production because customers remain largely opposed to genetically modified microorganisms. In the present study, we successfully isolated non-genetically modified yeasts (*S. cerevisiae*) with Orn overproduction. The non-genetically modified *S. cerevisiae* strains have been widely used in a variety of fermented foods, such as wine, beer, sake and bread. Therefore, strain A902-4 could be readily applied to sake, wine, and beer brewing.

Indeed, there were no significant differences in fermentation ability between strains K901, which is well commercialized in Japanese sake brewery, and A902-4 in the laboratory-scale sake brewing tests. Our results also showed that Orn levels were significantly increased in sake and sake cake brewed with strain A902-4 compared to strain K901. The large amount of Orn in sake is of particular interest. Although transporters involved in amino acids uptake have been extensively investigated, there have been much less studies on transporters responsible for excretion of amino acids. However, we believe that Orn is released actively into sake by the unidentified transporter rather than passively by cell lysis.

Orn has been reported to have some physiological functions, including improvement of sleep quality (Miyake et al., 2014) and an increase in serum growth hormone level (Demura et al., 2010). However, Orn was shown to have bitter taste (Harada et al., 2004). When we evaluated the effect of Orn on bitterness of the commercially available sake by the taste sensing system TS-5000Z (Intelligent Sensor Technology, Kanagawa, Japan), there was no significant differences between the sake without adding Orn (bitterness 0.80) and the sake even added 800 μg/mL of Orn (bitterness 0.89), suggesting that high-level Orn in sake has little adverse effect on the taste of sake. Thus, the Orn-accumulating strains have the potential for use in the production of Orn-rich dietary supplements by powderizing sake cake and yeast extracts by digesting yeast cells.

## 5. Conclusion

In this study, we successfully isolated a diploid sake yeast mutant A902-4 that accumulated not only Pro but also Orn by a conventional mutagenesis. Strain A902-4 had a novel mutation in the *ARG5,6* gene encoding the Thr340Ile variant of NAGK, which increases intracellular Pro and Orn levels remarkably. The Thr340Ile variant NAGK was extremely insensitive to Arg feedback inhibition. This is the first study to report the removal of feedback inhibition of NAGK activity, leading to overproduction of Orn in the industrial yeast cells. Furthermore, the Orn content in sake and sake cake brewed with strain A902-4 was

approximately 4–5 times higher than that of the parent strain K901. These results will contribute to the development of superior yeast strains for Orn overproduction.

## Declaration of competing interest

The authors declare there is no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2020.08.005.

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