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# Isolation of baker's yeast mutants with proline accumulation that showed enhanced tolerance to baking-associated stresses

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ABSTRACT

During bread-making processes, yeast cells are exposed to baking-associated stresses such as freeze-thaw, air-drying, and high-sucrose concentrations. Previously, we reported that self-cloning diploid baker's yeast strains that accumulate proline retained higher-level fermentation abilities in both frozen and sweet doughs than the wild-type strain. Although self-cloning yeasts do not have to be treated as genetically modified yeasts, the conventional methods for breeding baker's yeasts are more acceptable to consumers than the use of self-cloning yeasts. In this study, we isolated mutants resistant to the proline analogue azetidine-2-carboxylate (AZC) derived from diploid baker's yeast of *Saccharomyces cerevisiae*. Some of the mutants accumulated a greater amount of intracellular proline, and among them, 5 mutants showed higher cell viability than that observed in the parent wild-type strain under freezing or high-sucrose stress conditions. Two of them carried novel mutations in the *PRO1* gene encoding the Pro247Ser or Glu415Lys variant of γ-glutamyl kinase (GK), which is a key enzyme in proline biosynthesis in *S. cerevisiae*. Interestingly, we found that these mutations resulted in AZC resistance of yeast cells and desensitization to proline feedback inhibition of GK, leading to intracellular proline accumulation. Moreover, baker's yeast cells expressing the $PRO1^{P247S}$ and $PRO1^{E415K}$ gene were more tolerant to freezing stress than cells expressing the wild-type *PRO1* gene. The approach described here could be a practical method for the breeding of proline-accumulating baker's yeasts with higher tolerance to baking-associated stresses.


## 1. Introduction

Modern day food culture demands advanced technologies in the bread-making process, during which baker's yeast strains (usually *Saccharomyces cerevisiae*) face baking-associated stresses (Shima and Takagi, 2009), such as freeze-thaw, air-drying, and high-sucrose. Such stresses decrease the fermentation ability of baker's yeast and affect the properties of the dough resulting in poor taste and quality of the final products (Perez-Torrado et al., 2010; Ribotta et al., 2001, 2003). Due to controversy regarding the safety of genetically modified organisms in the food industry, there is a need to isolate baker's yeast strains that will permit safer genetic modifications, such as those using conventional mutagenesis or self-cloning (Steensels et al., 2014). However, consumers are still skeptical about the safety of using self-cloning microorganisms in the production of food for direct and/or indirect consumption (Kayabasi and Mucan, 2011).

Trehalose and proline are important compounds involved in the stress tolerance of baker's yeast (Shima and Takagi, 2009). We previously reported that self-cloning baker's yeast strains that accumulate proline are tolerant to various baking-associated stresses, including freeze-thaw, air-drying, and high-sucrose (Kaino et al., 2008; Sasano et al., 2010, 2012a, 2012b). Therefore, it might be possible to obtain genetically improved microorganisms by exposing wild-type cells to a conventional mutagen and selecting mutants with improved traits. A toxic analogue of proline, azetidine-2-carboxylate (AZC), enters cells via proline permeases. AZC causes misfolding and thermal instability of the proteins into which it is incorporated in a proline-competitive manner, and thereby inhibits cell growth (Trotter et al., 2002). However, by introducing mutation(s) favoring proline accumulation in the corresponding gene(s), the proteins of mutant cells incorporate proline and the cells become resistant to AZC. In this way, increased intracellular concentration of proline reduces the effect of toxic AZC.

In addition, proline has other protective functions in the cell. In bacterial and plant cells, proline acts as a salinity-related osmoprotectant, along with trehalose, glycerol, and betaine (Takagi, 2008). Exogeneous proline has a cryoprotective activity in yeast nearly equal to that of

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glycerol or trehalose (Takagi et al., 1997). Moreover, proline stabilizes proteins and the cell membrane (Hayat et al., 2012) and lowers the melting temperature ($T_m$) of DNA (Rajendrakumar et al., 1997). Proline has also been shown to scavenge reactive oxygen species (ROS) (Kaul et al., 2008; Smirnoff and Cumbes, 1989; Wang et al., 2009). Interestingly, proline significantly reduces the ROS level and increases the survival rate of yeast cells in the stationary phase under ethanol stress conditions (Takagi et al., 2016).

Proline-accumulating strains usually have a mutation on the *PRO1* gene, which encodes γ-glutamyl kinase (GK). GK is the rate-limiting enzyme of proline biosynthesis from glutamate, and its activity is regulated allosterically by the end product proline (Sekine et al., 2007) (Fig. 1). The majority of proline-accumulating strains have been obtained by expressing GK variants that are less-sensitive to feedback inhibition by proline (Morita et al., 2003; Sekine et al., 2007).

In the present study, we isolated for the first time AZC-resistant mutants derived from a baker's yeast strain. Some of the mutants were found to accumulate intracellular proline and to exhibit increased tolerance to freeze-thaw and high-sucrose stresses. We also discuss the mechanism of proline accumulation by the above-described mutant with mutations in the *PRO1* gene. In addition, to examine the effect of the *PRO1* mutations on GK properties and stress tolerance, we constructed baker's yeast strains expressing the GK variants and analyzed their characteristics.

## 2. Materials and methods

### 2.1. Strains and culture media

We used the diploid Japanese baker's yeast strain of *S. cerevisiae* 3346/3347. *S. cerevisiae* cells were grown in a nutrient rich medium YPD (1% yeast extract, 2% peptone, and 2% glucose), synthetic defined medium SD + Am or SD-N + All (2% glucose, 0.67% yeast nitrogen base without ammonium sulfate and amino acids [Difco Laboratories, Detroit, MI, USA], and 0.5% ammonium sulfate or 0.5% allantoin, respectively), and cane molasses medium (5.88% NEO MOLASSEST [EM laboratory, Shizuoka, Japan], 0.193% urea, and 0.046% $KH_2PO_4$). The composition of the pre-fermentation medium for freezing stress and the liquid fermentation medium for high-sucrose stress was the same as that described in previous reports (Sasano et al., 2012a, 2012b), respectively. The *S. cerevisiae* recombinant strains were grown in YPD medium or SD-N + All containing 200 μg/ml G418. *Escherichia coli* recombinant strains were grown in Luria-Bertani (LB) complete medium containing 100 μg/ml ampicillin. When necessary, 2% agar was added to solidify the medium.

### 2.2. Isolation of AZC-resistant mutant

AZC-resistant mutants were obtained by conventional mutagenesis. Random mutations were induced by treatment with 5% ethyl methanesulfonate (EMS) (Rose and Broach, 1991). The mutagenized cells were spread onto SD + Am agar plates containing 2 mg/ml AZC (Bachem, Bubendorf, Switzerland). After incubation at 30 °C for 3 days, the resulting colonies were collected and tested for AZC resistance and amino acid production.

### 2.3. Spot test for AZC resistance

Yeast cells were cultured in 3 ml of YPD + G418 medium. After overnight incubation at 30 °C with rotary shaking, cells corresponding to an $OD_{600}$ of 1.0 were collected, washed twice, suspended in 1 ml of water, and serially diluted. 5 μl of diluted cell suspensions were spotted on SD-N + All agar plates containing 2 mg/ml or 5 mg/ml AZC and incubated at 30 °C for 2–7 days. Transcription of the *PUT4* gene encoding a proline specific permease is repressed by ammonia (Andréasson et al., 2004; Jauniaux et al., 1987) and hence allantoin was used as the nitrogen source to allow uninterrupted influx of AZC.

### 2.4. Measurement for intracellular amino acid content

Yeast cells were cultured in 3 ml of YPD or cane molasses medium. After overnight cultivation at 30 °C, 1 ml of the cultured sample was washed and inoculated into 30 ml of the above medium. After cultivation for 48 h at 30 °C with rotary shaking, cells corresponding to an $OD_{600}$ of 10 were collected, washed twice and suspended in 0.5 ml of distilled water. Intracellular amino acids in cell suspension were extracted by boiling for 20 min at 100 °C. After centrifugation (15,000 × g, 5 min, 4 °C), amino acid content in each supernatant was subsequently quantified with amino acid analyzer (AminoTac JLC-500/V; JEOL, Tokyo, Japan). The content of each amino acid was expressed as a percentage of dry weight.

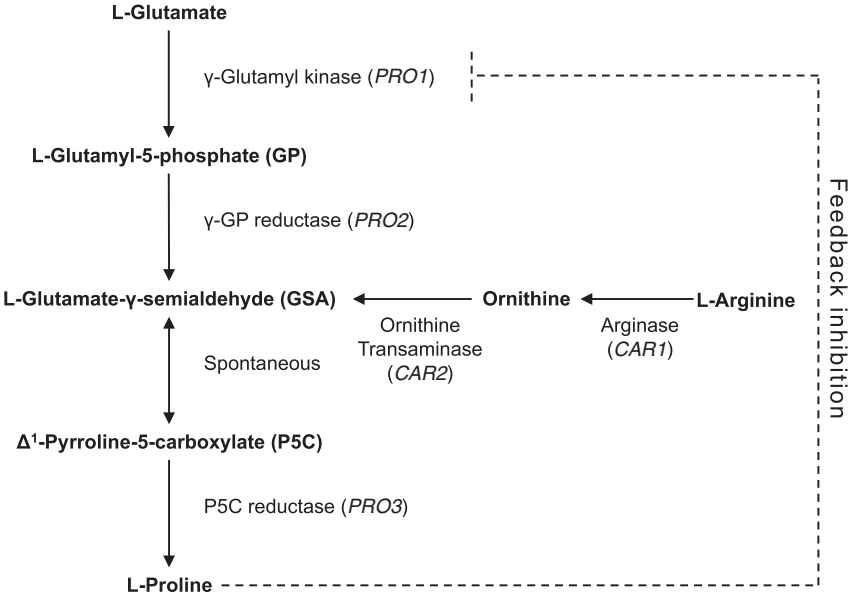


**Fig. 1.** Proline synthetic pathway in *Saccharomyces cerevisiae*. Genes encoding particular enzymes are shown in parentheses.