Clarification of inhibitory mechanism of proline utilization in fermentations and its application

Akira NISHIMURA

Graduate School of Science and Technology, Nara Institute of Science and Technology

Research objective

Yeasts play an important role in ethanol production and dictating the aroma and taste of alcoholic beverages. Thus, the quality of alcoholic beverages strongly depends on the metabolic characteristics of yeast cells. In the wine-making process, grapes must contain a sufficient quantity of sugar for the maximal growth of wine yeasts. In contrast, the total nitrogen content of the grapes must range from 60 to 2,400 mg/L, which limits yeast growth. Therefore, nitrogen availability is an essential parameter of wine fermentation.

Proline is the predominant amino acid in grapes and is considered a potentially significant and effective source of nitrogen. However, the wine yeast *Saccharomyces cerevisiae* cannot utilize proline during wine fermentation[1)]. This often leads to nitrogen deficiency during fermentation and proline accumulation in wine. To prevent nitrogen deficiency, ammonium salts, such as diammonium phosphate, are frequently supplied during the fermentation processes. Such additives can impact yeast metabolism and thus, influence the quality of the wine by altering the aroma and taste profiles. Moreover, supplementation with ammonium salt causes the accumulation of ethyl carbamate, a carcinogen. In addition, excess proline in wine has negative effects on wine quality, because residual proline leads to a decrease in the sweetness and acidity of wine. Hence, a yeast strain that can utilize proline is a promising candidate for overcoming nutrient-related fermentation problems, thereby improving wine fermentation ability and wine quality.

Methods

1. Strains

The wine yeast wild-type strain (provided by Dr. Walker, University of Adelaide), a proline auxotrophic strain (*pro3*Δ/*pro3*Δ), and a *cdc25*^{Glu916*} mutant strain (*cdc25*^{Glu916Stop}/ *cdc25*^{Glu916Stop}) were used in this study.

2. Measurement of residual amino acids

The yeast strains were inoculated into white grape (WG) medium starting from an optical density at 600 nm (OD_{600}) of 1.5. After incubation under static conditions at 30°C for the durations indicated in the figure legends, the supernatants were collected by centrifugation. The residual amino acid content of each supernatant was determined using an amino acid analyzer (JLC-500/V; JEOL, Tokyo, Japan).

3. Isolation of proline-utilizing mutants under WG medium

Strain *pro3*Δ/*pro3*Δ was plated on WG medium. After 3 days at 30°C, the two colonies were

picked, and named P1 and P2. For whole-genome sequencing, genomic DNA was extracted from cells using a Dr. GenTLE (from yeast) High Recovery Kit (Takara Bio, Kusatsu, Japan). Libraries for sequencing analysis were prepared using an NEB Next Ultra DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA), and paired-end reads of 150 bp were produced using a NovaSeq 6000 (Illumina, San Diego, CA, USA). Sequencing was performed by a commercial DNA sequencing service (Rhelixa, Tokyo, Japan).

4. cAMP determination

Each strain was suspended in WG medium and the cells were collected by centrifugation at the time points indicated in the figure legends. Metabolites were extracted from the cells adding trichloroacetic acid (final concentration 5%). cAMP levels in the extracts were determined using a cAMP EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Values were normalized to OD_{600} units.

5. Detection of proteins phosphorylated by protein kinase A

Protein kinase A (PKA)-phosphorylated proteins were identified by western blotting using an anti-phosphorylated PKA consensus RRxS/T rabbit antibody (Cell Signaling Technology, Danvers, MA, USA).

Results

We performed genetic screening to identify genes involved in the inhibition of proline utilization. By screening with *pro3*Δ/*pro3*Δ and WG medium, we obtained two spontaneous mutants (which we named P1 and P2) that were able to grow on WG medium. The growth phenotypes of P1 and P2 were confirmed in liquid WG medium. Importantly, P1 and P2 clearly consumed proline under static conditions in WG medium, whereas the WT/WT and *pro3*Δ/*pro3*Δ strains did not consume proline. Whole-genome sequencing analysis revealed that both P1 and P2 had a T nucleotide at position 2746, only at the *CDC25* locus encoding a guanine nucleotide exchange factor (GEF) of Ras1/2, which is involved in the glucose response. In contrast, *pro3*Δ/*pro3*Δ and laboratory yeast strains with publicly available genomic information (*Saccharomyces* Genome Database: https://www.yeastgenome.org/) have a G nucleotide at the same position. This G to T mutation (2746 G>T) led to the occurrence of a stop codon at position 916 (Glu916*), indicating that P1 and P2 have a homozygous nonsense mutation in *CDC25*. *cdc25*^{Glu916*} encodes a Cdc25 variant with a GEF domain deletion, suggesting that Cdc25 Glu916* is a loss-of-function mutant. Next, we examined the effects of the *cdc25*^{Glu916*} mutation on Ras/PKA signaling in WG medium. cAMP levels in the WT/WT strain transiently increased for up to 10 min upon transfer to WG medium, but cAMP levels in the *cdc25*^{Glu916Stop}/ *cdc25*^{Glu916Stop} strain did not. The phosphorylation levels of PKA substrates in the WT/WT strain increased upon transfer to WG medium. The *cdc25*^{Glu916*} mutation resulted in no significant change in the phosphorylation

levels of PKA substrates upon transfer to WG medium. Finally, we determined proline consumption by the WT/WT and *cdc25*^{Glu916Stop}/ *cdc25*^{Glu916Stop} strains. The WT/WT strain did not consume proline in WG medium. In contrast, the *cdc25*^{Glu916Stop}/ *cdc25*^{Glu916Stop} strains showed clear consumption of proline. Activation of the Ras/PKA pathway inhibits proline utilization in wine yeast (Figure 1)[2]. Herein, we propose that crosstalk occurs between carbon metabolism and proline utilization.

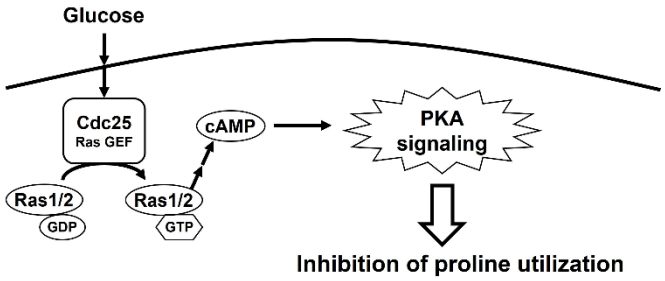

Figure 1 Regulation of proline utilization by the PKA pathway.
Activation of the Ras/PKA pathway via Cdc25 by glucose leads to the inhibition of proline utilization.

Conclusion

Wine is a traditional fermented beverage brewed with wine yeast (most commonly *S. cerevisiae*). Product innovation is a vital source of competitive advantage, and wine is no exception to this principle. Therefore, winemakers continue to pursue new wine yeast strains to meet the demand for high-quality wines. Proline is the predominant amino acid in grape must, but is poorly utilized by yeast during the wine-making processes. This phenomenon negatively affects winemaking quality and productivity. Yeast cells have been known for more than 50 years to consume little proline during wine fermentation; however, the detailed underlying mechanisms remain unclear. Our data suggest that the carbon sources present during wine fermentation activate PKA signaling via the Cdc25/Ras pathway, which in turn, leads to the inhibition of proline utilization by yeast. In short, they suggested the existence of crosstalk between carbon and proline metabolism. Our results hold promise for the development of wine yeast strains that are capable of efficiently assimilating proline during fermentation.

References

1) Valero, E., Millán, C., Ortega, J. M., and Mauricio, J. C. (2003) Concentration of amino acids in wine after the end of fermentation by *Saccharomyces cerevisiae* strains. *J. Sci. Food Agric.* **83**: 830-835.

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