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Defective quiescence entry promotes the fermentation performance of bottom-fermenting brewer's yeast

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One of the key processes in making beer is fermentation. In the fermentation process, brewer's yeast plays an essential role in both the production of ethanol and the flavor profile of beer. Therefore, the mechanism of ethanol fermentation by of brewer's yeast is attracting much attention. The high ethanol productivity of sake yeast has provided a good basis from which to investigate the factors that regulate the fermentation rates of brewer's yeast. Recent studies found that the elevated fermentation rate of sake *Saccharomyces cerevisiae* species is closely related to a defective transition from vegetative growth to the quiescent (G_0) state. In the present study, to clarify the relationship between the fermentation rate of brewer's yeast and entry into G_0, we constructed two types of mutant of the bottom-fermenting brewer's yeast *Saccharomyces pastorianus* Weihenstephan 34/70: a *RIM15* gene disruptant that was defective in entry into G_0; and a *CLN3ΔPEST* mutant, in which the G_1 cyclin Cln3p accumulated at high levels. Both strains exhibited higher fermentation rates under high-maltose medium or high-gravity wort conditions (20° Plato) as compared with the wild-type strain. Furthermore, G_1 arrest and/or G_0 entry were defective in both the *RIM15* disruptant and the *CLN3ΔPEST* mutant as compared with the wild-type strain. Taken together, these results indicate that regulation of the G_0/G_1 transition might govern the fermentation rate of bottom-fermenting brewer's yeast in high-gravity wort.

[**Key words:** Ethanol fermentation; Brewer's yeast; *Saccharomyces pastorianus*; Quiescent state; G_0 entry; *RIM15*; *CLN3*]

In the process of beer-making, brewer's yeast metabolizes fermentable sugars into ethanol and CO_2, while producing other byproducts that influence the beer's flavors and aromas such as estery, molty and yeasty (1–5). Faster fermentation facilitates an increase in ethanol yield and contributes to shorter fermentation periods. By contrast, a lower fermentation performance can cause undesirable final beer quality owing to increasing residual sugars and a low ethanol content (6–8). In addition, to reduce energy and production costs and time, a brewer's yeast suited to high-gravity brewing is desirable (7,9). However, the control mechanism underlying the fermentation performance of brewer's yeast remains unclear.

Many studies have been carried out to identify factors that determine the fermentation performance of brewer's yeast. For example, a higher temperature improves the fermentation rate by accelerating nitrogen uptake (10). Supplementation with metal ions, such as zinc and magnesium, increases ethanol production by positively promoting yeast growth (11–14). Oxygen, osmotic pressure, free amino nitrogen and lipids are also known to influence the fermentation rate of brewer's yeast (15–19). However, these studies have all focused on the fermentation conditions or wort composition, and there is little research on the mechanism underlying the control of fermentation by brewer's yeast itself.

The elevated fermentation rate and high ethanol productivity of sake *Saccharomyces cerevisiae* strains form a good basis with which to elucidate the factors regulating the fermentation rate of brewer's yeast. Sake is a traditional Japanese alcohol beverage brewed primarily from rice and using koji and yeast *S. cerevisiae*. It is known that sake yeast strains produce much more ethanol (≥20% (v/v)) during sake fermentation. Recent studies have revealed that a defective transition to the G_0 phase is one of the reasons why sake yeast strains produce high concentrations of ethanol (20–24). In *S. cerevisiae*, the Greatwall-like protein kinase Rim15p plays the role of master regulator in triggering the G_0 program by stimulating G_0 transcription factors such as Msn2/4p and Gis1p (25–27). Modern sake yeast strains have a common loss-of-function frameshift mutation in the *RIM15* gene, namely $rim15^{5055insA}$ (20,21); as a result, they exhibit the phenotype of defective entry into G_0 phase, which may contribute to maintaining cell cycle progression and the high fermentation rate. This hypothesis is supported by data showing that deletion of *RIM15* in the laboratory strain BY4743 leads to defects in G_0/G_1 arrest and an increasing fermentation rate (20).

Furthermore, it has been reported that the expression of G_1 cyclin *CLN3* mRNA in the sake yeast strain Kyokai no. 7 (K7) is elevated during fermentation as compared with a laboratory strain (22). In *S. cerevisiae*, Cln3p together with the Cdc28p cyclin-

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dependent kinase (CDK) is the most upstream regulator of the genes involved in the G_1/S transition (28–31). Previous studies have revealed that Cln3p is highly unstable and degraded in a PEST-rich C-terminal sequence-dependent manner under conditions of phosphate and nitrogen deprivation (32,33). In contrast, Cln3-1p, which is encoded by a mutated *CLN3* gene in the *WHI1-1* dominant mutant and lacks the C-terminal PEST-rich regions, is stable and thus accelerates the G_1/S transition (30). Notably, the phenotype of the *WHI1-1* mutant is similar to that of the sake yeast K7 strain. Moreover, the *WHI1-1* (*CLN3ΔPEST*) mutation has been shown to improve the fermentation rate of the laboratory strain BY4743 (22). These data suggest that the loss-of-function mutation in *RIM15* gene and the highly expressed *CLN3* gene probably contribute to the high fermentation rate of sake yeast. However, it is not known whether the altered G_0/G_1 transition also influences the fermentation rate of bottom-fermenting brewer's yeast, which is used to brew lager-type beers around the world and thus attracts much attention in terms of its fermentation properties.

The aim of the present study was to examine whether the fermentation rate of bottom-fermenting brewer's yeast can be improved by applying previous knowledge gained from sake yeast.

MATERIALS AND METHODS

Yeast strains The bottom-fermenting brewer's yeast strain *Saccharomyces pastorianus* W34/70 was the stock strain. The laboratory *S. cerevisiae* strain BY4743 and its Δ*rim15::kanMX* disruptant were provided by Euroscarf; BY4743 *CLN3ΔPEST::kanMX* was constructed in a previous study (22). The laboratory *S. cerevisiae* strain X2180 was provided by the American Type Culture Collection. All yeast strains were cultured in YPD medium [1% yeast extract, 2% Bacto-peptone (Difco, Sparks, MD, USA), 2% glucose] at 25°C, unless specified otherwise.

Mutants of X2180-1A or W34/70 were constructed by using a PCR-based method (34). The primers used in this study are shown in Table 1. To disrupt the *RIM15* gene in the X2180-background, the primers RIM15-DF and RIM15-DR and the plasmid pAG25 (34) were used. Correct disruption was confirmed by PCR using the primers SC RIM15-F and SC RIM15-R. To disrupt two (or more) copies of the *S. cerevisiae* (SC)-type *RIM15* gene of W34/70, DNA fragments were amplified by PCR using the genomic DNA of BY4743 Δ*rim15::kanMX* or X2180-1A Δ*rim15::natMX* as a template and the primers SC RIM15-F and SC RIM15-R (Fig. 1A). To delete the PEST region in the SC-type *CLN3* gene of the W34/70 strain, a DNA fragment was amplified by PCR using the genomic DNA of BY4743 *CLN3ΔPEST::kanMX* as a template and the primers SC CLN3-F and SC CLN3-R (Fig. 1B). The resulting DNA fragments were used for transformation. Sequence analysis was performed by using a CEQ8000 DNA analysis system (Beckman Coulter).

Transformation of W34/70 was performed by the lithium acetate/polyethylene glycol method (35). Transformants were selected on a YPD plate containing 500 μg/mL of G-418 or 100 μg/mL (X2180-1A) or 200 μg/mL (W34/70) of nourseothricin.

Fermentation test Fermentation tests in synthetic medium (50-mL scale) were performed as follows: pre-cultured yeast cells in YPD overnight were washed by distilled water and inoculated in 50 mL of 12% or 20% maltose medium (0.67% yeast nitrogen base without amino acids, 1% yeast extract, and 12% or 20% maltose) at an initial cell concentration of OD660 = 2.0. The cells were incubated at 15°C for 7 days with shaking at 90 rpm. Fermentation rate was monitored by measuring the volume of carbon dioxide produced using a Fermograph II instrument (Atto) (36). Ethanol concentration was measured by gas chromatography (GC-17A, Shimadzu, Japan). Apparent extract was determined by using a density/specific gravity meter (DA-510, Kyoto Electronics Manufacturing).

TABLE 1. PCR primers used in this study.

Primer	Sequence
RIM15-DF	5′-CTCTTGCCTCATTTGATAGAATAGATAAGCC CAGTAGAGGAAGACCGTACGCTGCAGGTCGAC -3′
RIM15-DR	5′-TTTTTATTCAGTTATTTTTTTTAATTATCTTTATCTT AAAATTTAATCGATGAATTCGAGCTCG -3′
SC RIM15-F	5′-AAGTTGTTGTTCGTATCACAGC-3′
SC RIM15-R	5′-CTCTAACAAAGGAGAATATATATACG-3′
SC CLN3-F	5′-TCAACCTCGACTTTGTTCCT-3′
SC CLN3-R	5′-GTAATTCTATGCAAATGTAATATAA-3′
SC CLN3 Check-F	5′-GTCTCTCCCACACAGAAAGG-3′

Fermentation tests in high-maltose medium (1-L scale) were performed as follows: yeast strains were inoculated in 10 mL of wort and cultivated at 25°C for 1 day with shaking at 90 rpm. This culture liquid was added to 500 mL of aerated wort and cultivated at 15°C for 7 days without shaking. Pre-cultured yeast cells were inoculated in 1 L of well-aerated high-maltose medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.25% yeast extract, 20% maltose). The initial yeast concentration was 2×10^7 cells/mL and fermentation proceeded at 15°C for 18 days without shaking.

The high-gravity wort fermentation test (1-L scale) was performed as follows: yeast strains were inoculated in 10 mL of wort and cultivated at 25°C for 1 day with shaking at 90 rpm. This culture liquid was added to 500 mL of aerated wort and cultivated at 15°C for 7 days without shaking. Pre-cultured yeast cells were inoculated in 1 L of well-aerated 20° Plato wort adjusted by addition of maltose. The initial yeast concentration was 2×10^7 cells/mL and fermentation was performed at 15°C for 11 days without shaking.

Fermentation rates in both high-maltose medium (1-L scale) and high-gravity wort (1-L scale) were evaluated by measuring the apparent extract using a density/specific gravity meter (DA-510, Kyoto Electronics Manufacturing). Ethanol concentration was measured by using a biosensor (BF-5, Oji Scientific Instruments, Japan).

Flow cytometric analysis Rapamycin treatment tests were performed as described previously (20). The cell cycle stage of yeast cells was analyzed by flow cytometry. Approximately 1×10^7 yeast cells were withdrawn from each fermentation sample. Cells were fixed in 1 mL of 70% ethanol, washed with 1 mL of 50 mM Tris–HCl (pH 7.6), resuspended in 400 μL of RNase solution [1 mg/mL RNase A (Nacalai Tesque) in 50 mM Tris–HCl (pH 7.6)], incubated at 37°C for 120 min, washed with 1 mL of 50 mM Tris–HCl (pH 7.6) again and resuspended in 1 mL of proteinase K solution [0.5 mg/mL proteinase K (Nacalai Tesque) in 50 mM Tris–HCl (pH 7.6)]. After incubation at 4°C overnight, cells were centrifuged and resuspended in 1 mL of 50 mM Tris–HCl (pH 7.6). The cell suspension was sonicated by an ultrasound disintegrator (Handy Sonic UR-20P; Tomy Seiko), and 500 μL was then mixed with 500 μL of propidium iodide (PI) solution [200 μg/mL of PI in 50 mM Tris–HCl (pH 7.6)] and was analyzed by a FACSCalibur instrument (BD Biosciences).

RESULTS

Construction of an W34/70 SC-type *RIM15* gene disruptant and SC-type *CLN3ΔPEST* mutant The bottom-fermenting yeast *S. pastorianus* is thought to be a natural hybrid of *S. cerevisiae* (SC) and *S. eubayanus* (SB) (37,38). The DDBJ/EMBL/GenBank accession numbers for the DNA nucleotide sequences of W34/70 used in this study are as follows: SC-type *RIM15* BBYY01000119 17823-23144; SC-type *RIM15* BBYY01000681 59-5320; SC-type *CLN3* BBYY01000715 3331-5073; SB-type *CLN3* BBYY0100024 41786-40047. In this study, we constructed an SC-type *RIM15* disruptant and *CLN3ΔPEST* mutant by means of the strategy shown in Fig. 1. Since previous study showed SC-type *RIM15* and *CLN3* genes are to be effective in sake yeast *S. cerevisiae* (20,22), we picked up only SC-type genes.

To confirm the disruption of two (or more) copies of the SC-type *RIM15* genes of W34/70, we performed genomic PCR using the primers SC RIM15-F and SC RIM15-R. The predicted DNA sizes of PCR products of the wild-type SC-type *RIM15* gene, the *kanMX* gene and the *natMX* gene were approximately 5.3 kb, 1.6 kb and 1.2 kb, respectively. We verified that both the SC-type *RIM15* genes were correctly replaced by the *kanMX* and *natMX* genes from the results of the amplified PCR products (Fig. 2A).

To confirm deletion of the PEST region in one of the SC-type *CLN3* genes of W34/70, we performed genomic PCR using the primers SC CLN3 Check-F and SC CLN3-R (Table 1). Two PCR products, 2.0 kb and 2.5 kb, were expected to be the SC-type *CLN3* gene and the SC-type *CLN3ΔPEST* gene, respectively. These results indicated that the constructed strain had a mutated *CLN3ΔPEST* gene in one of the two (or more) SC-type *CLN3* alleles (Fig. 2B). We confirmed that there were no mutations in the other regions of the SC-type *CLN3* genes (data not shown).

Effect of disruption of *RIM15* or deletion of the PEST-rich region in *CLN3* on the cell cycle profile of W34/70 To clarify whether the constructed strains showed characteristics of hindered G_0/G_1 phase transition similar to sake yeast (20,22), we evaluated

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