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# Study of the High Ethanol Productivity of Sake Yeast by Comprehensive Cellular Morphological Analysis

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## Research aims

Sake yeast strains, which belong to the budding yeast species *Saccharomyces cerevisiae*, produce much more ethanol during sake fermentation than any other type of *S. cerevisiae* strain. To elucidate the molecular mechanisms responsible for their excellent fermentation properties, we here investigated sake yeast-specific morphological traits using the CalMorph system[1], which has been developed for high-dimensional and quantitative morphological analysis of yeast cell populations. This analysis led to identification of a novel genetic determinant for ethanol production in *S. cerevisiae*.

## Methods

The sake yeast strains Kyokai no. 6, 7, 9, and 10 and a laboratory strain X2180 were grown to the logarithmic phase in YPD medium and were then fixed with formaldehyde. After triple staining of yeast cell wall mannoproteins, actin, and nuclear DNA, cells were observed under the fluorescent microscope, and the microscopic cellular images were automatically processed using the CalMorph system.

For fermentation tests, yeast cells were inoculated into 20% glucose-containing YPD medium at a final $OD_{660}$ of 0.1, and were then further incubated at 30°C without shaking. For sake fermentation assays, a sake mash was prepared by mixing 40 g pre-gelatinized rice, 10 g dried koji (rice cultivated with *A. oryzae*), 20 $\mu$l 90% lactic acid, and 80 ml water containing yeast cells at a final $OD_{660}$ of 1.0, and was then incubated at 15°C without shaking. Fermentation was monitored by measuring the volume of evolved carbon dioxide using Fermograph[2].

To determine cellular DNA contents, yeast cells were fixed in ethanol, suspended in a propidium iodide solution, and analyzed using a flow cytometer. Gene-specific quantitative real-time PCR (qRT-PCR) was performed to quantify *CLN3* mRNA. The actin-encoding gene *ACT1* was used as an internal control.

## Results

Our high-throughput cytological analysis using CalMorph revealed that sake yeast cells exhibit specific morphological features in the logarithmic phase: Both the ratio-of-no-bud (C119) and long-axis-length-of-mother-cell-on-nuclear-A1B (C103_A1B) parameters were significantly lower in all of the sake strains than in X2180 (Fig. 1). Since these morphological traits have also been typically observed in the mutants that cannot effectively repress the G1/S transition, we hypothesized that the G1 progression might be accelerated in sake yeast.

Under fermentative conditions, is sake yeast defective in cell cycle regulation? To test this, we performed fermentation tests using the sake and laboratory strains in 20% glucose-containing YPD medium to analyze the cellular DNA contents during fermentation. We found that the cell population of sake yeast with a G1 DNA content was smaller than that of laboratory yeast throughout the fermentative phase, as well as in the logarithmic growth phase (Fig. 2A). We also discovered elevated expression of the G1 cyclin gene *CLN3* in sake yeast under the fermentation condition (Fig. 2B). These results indicate that laboratory yeast exhibited efficient G1 arrest during the fermentative phase, whereas some of the sake yeast cells failed to maintain G1 arrest until fermentation termination[3].

To elucidate the mechanism causing the defects of sake yeast in cell cycle control, the mutations in the genes related to G1 progression were thoroughly searched by utilizing the genome sequence data of a sake strain Kyokai no. 7 that we recently reported[4]. Consequently, we identified a novel frameshift mutation, $rim15^{5055insA}$, causing dysfunction of the Rim15p protein kinase, which is responsible for effective G1 arrest upon growth termination[5]. Among various industrial yeast strains (e.g., wine and beer yeast), sake strains including Kyokai no. 6, 7, 9, and 10 specifically contained this mutation. Furthermore, expression of a functional *RIM15* gene recovered an effective G1 arrest in sake yeast (data not shown), demonstrating that the $rim15^{5055insA}$ mutation accounts for the defective cell cycle control in sake yeast.

Fermentation tests using a Δ*rim15* gene disruptant and a $rim15^{5055insA}$ mutant in the laboratory strain BY4743 background revealed how Rim15p-mediated G1 progression control affects the fermentation property. Loss of the Rim15p functions significantly enhanced the fermentation rate of the parental strain (Fig. 3)[5]. These *rim15* mutants exhibited markedly improved fermentation rates also in small-scale sake brewing tests: The ethanol concentration after 20 days of sake fermentation was higher in the finished sake made from the Δ*rim15* disruptant (17.03± 0.44%) and that from the $rim15^{5055insA}$ mutant (16.77±

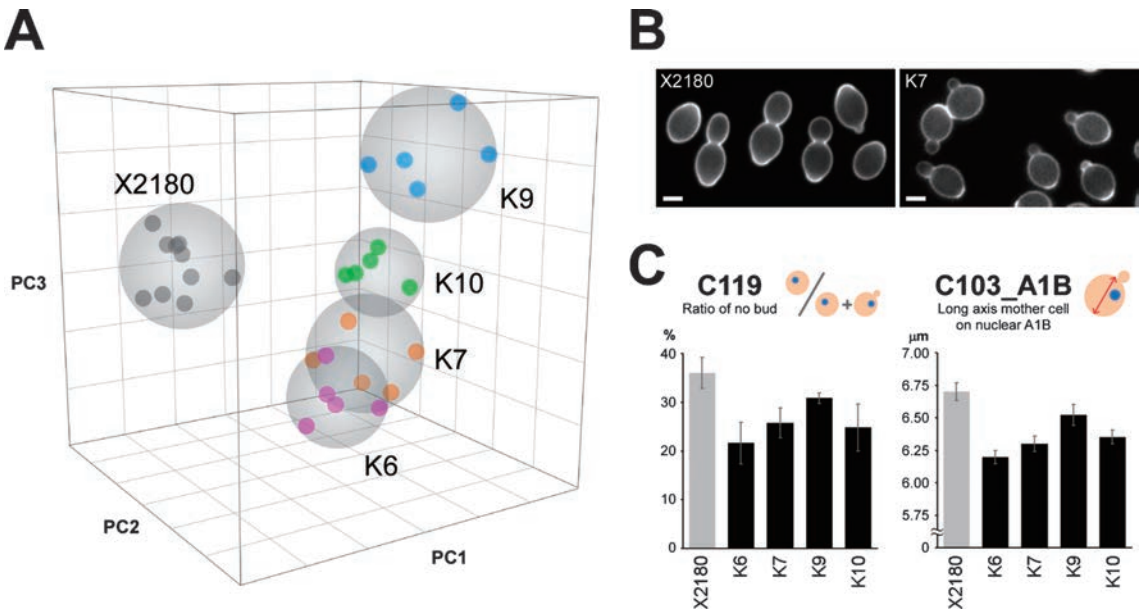


Fig. 1. Morphological traits of the sake yeast strains. (A) Three-dimensional PCA mapping of the CalMorph data. Colored dots correspond to data from individual experiments (n>200 cells). Sake yeast strains Kyokai no. 6, 7, 9, and 10 (K6, K7, K9, and K10) and a laboratory strain X2180 were used. (B) Representative cell morphology of a sake and a laboratory yeast. Bar; 3 mm. (C) Morphological parameters that showed significant differences between sake and laboratory yeast.

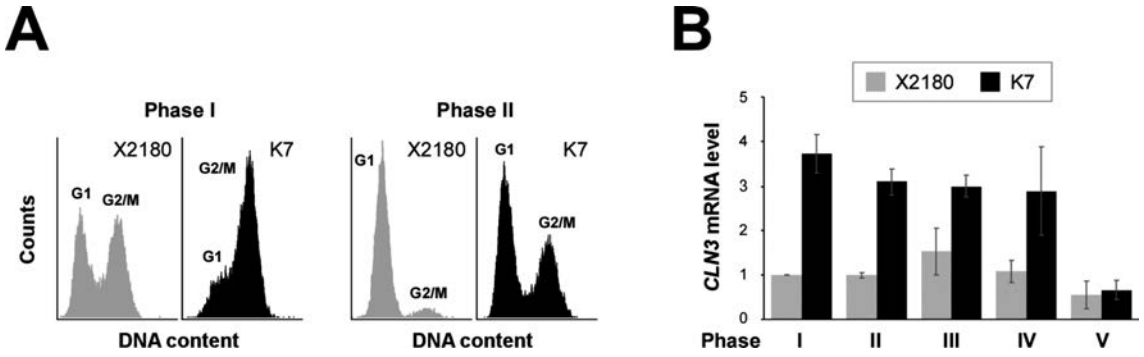


Fig. 2. Cell cycle-related analysis under a fermentative condition. Phase I: logarithmic growth phase, Phases II–IV: fermentative phases; Phase V: fermentation termination. (A) DNA contents. (B) Analysis of *CLN3* mRNA levels using qRT-PCR. Relative expression levels are displayed as fold differences compared to the levels induced in X2180 (phase I), using *ACT1* as a reference gene.

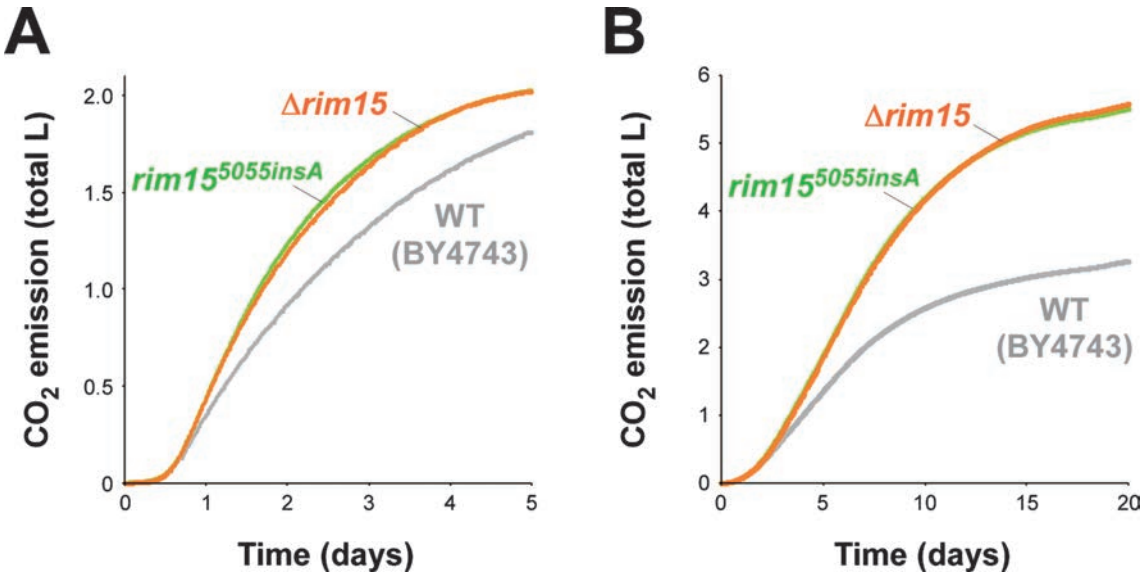


Fig. 3. Improvement of the fermentation rate by dysfunction of Rim15p. (A) Fermentation tests in 20% glucose-containing YPD medium. (B) Small-scale sake fermentation tests.

0.18%) than that from wild-type BY4743 (11.19±0.17%). Taken together, these results clearly demonstrated that dysfunction of Rim15p caused by the sake yeast-specific *rim15*$^{5055insA}$ mutation leads to a striking increase of ethanol production.

## Conclusion

The cellular morphological analysis of sake yeast enabled us to find its novel phenotype related to defective cell cycle control. Furthermore, we revealed that the *rim15*$^{5055insA}$ mutation have played a pivotal influential role in accelerated G1 progression and increased fermentation rate that are both characteristic of sake yeast strains. Identification of multiple genetic determinants for the fermentation properties of sake yeast[5–7] will provide significant insights into artificial modification of the yeast metabolic profiles. In future studies, the molecular mechanism on how the Rim15p-mediated cell cycle regulatory system controls fermentation and ethanol production should be intensively investigated.

## References


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