

A research on mitochondria-targeted breeding of a novel brewery yeast

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Research aims

Since mitochondria have been described to be responsible for oxidative respiration and brewery yeast is exposed to an environment in which oxidative respiration does not occur during alcoholic fermentation, the role of yeast mitochondria during alcoholic fermentation has not been described in detail. However, I have demonstrated that yeast mitochondria do exist throughout brewing of sake, the Japanese traditional rice wine, and that their structures undergo fragmentation during sake brewing¹⁾. I was inspired to apply these novel findings to the brewing technologies.

Methods

1. Comparison of gene expression change across diauxic shift in WT cells and mitochondria-defective mutant cells

Yeast strain JK93d- α was used as the parental strain. Yeast cells were routinely grown in Difco YPD broth with shaking at 30°C. RNA was isolated from cell pellets using the RNeasy RNA isolation kit (Qiagen) following the enzymatic lysis protocol. Microarray analysis was carried out essentially as described in the Affymetrix Expression Analysis Technical Manual. Microarray data were derived from two independent experiments for WT cells and one experiment for respiration-defective petite cells. Absolute analysis was conducted for each analysis with scaling to a target value of 2500 to facilitate comparison between experiments. In experiments shown in Fig. 1, genes whose expression at 24 h in WT was greater than 300 were selected. Among the selected genes, the genes whose expression ratio of WT-24 h/WT-4 h was more than 2-fold were further selected. Mitochondrial DNA was depleted as follows. Logarithmically grown yeast cells were diluted to 2×10^6 cells/ml in 1% yeast extract, 2% peptone, 2% dextrose, 50 mM potassium phosphate, pH 6.24. Ethidium bromide was added to a final concentration of 50 μ M, and the cultures were incubated at 30°C in the dark for 8 h with shaking and then plated onto YPD or YPGE medium to confirm inability to grow on nonfermentable medium.

2. Mitochondrial-morphology-targeted breeding of brewery yeast strains

Sake yeast Kyokai No. 7 was purchased from the Brewing Society of Japan, Tokyo. The haploid strain K7H868 was obtained by sporulating Kyokai No. 7 and screening for a haploid strain with a fermenting ability similar to the

parental diploid strain. The yeast cells were incubated in 2% Bacto-peptone, 1% Bacto-yeast extract (Beckton Dickinson, USA) and 2% glucose at 30°C with shaking. Cells harboring mitochondria-targeted GFP were grown in a synthetic medium containing a 0.67% yeast nitrogen base without amino acids (Beckton Dickinson), a 0.2% complete supplement mixture without leucine (MP Biomedicals, USA) and 2% glucose at 30°C with shaking. The content of organic acids was determined using a high-performance liquid chromatograph equipped with a conductivity detector and a column Shim-pack SPR-H (Shimadzu). Disruption of *FIS1* gene in K7 haploid strain was performed by transformation using a disruption cassette amplified with suitable primers, and selection on 100 μ g/ml nourseothricin-containing plates by standard techniques. Disruption of *FIS1* was confirmed by PCR analysis and staining with a mitochondrial dye (50 nM rhodamine 123, Invitrogen, USA). In order to transform mitochondria-targeted GFP, *LEU2* was disrupted using a disruption cassette amplified with suitable primers. The transformation was performed by standard techniques and colonies were plated onto YPD plates containing 500 μ g/ml G418. For visualization of mitochondria, strain RAK1536, pYX142-mitoGFP, and pRS413GPD-mtGFP were used. They were kind gifts from Professor Rinji Akada of Yamaguchi University, Dr. Koji Okamoto of

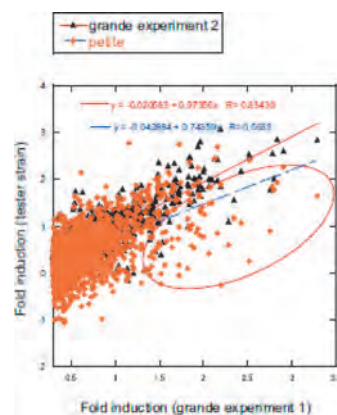


Fig. 1. Comparison of gene expression change across the diauxic shift between respiration-competent WT (grande) cells and respiration-defective petite cells.

The genes that were up-regulated more than 2-fold in WT experiment 1 at 24 h relative to 4 h were selected. The \log_{10} value of induction ratio of gene expression at 24 h to that at 4 h in WT experiment 2 and petite experiment were plotted against that in WT experiment 1.

National Institute of Basic Biology, and Professor Shaw of Utah University, respectively. Sake brewing was performed by mixing 2×10^9 cells of yeast with 60 g of dried gelatinized rice, 23 g of dried gelatinized koji, 45 μ l of lactic acid, 200 ml of water and incubating the mash at 15°C for 14 days.

Results

1. Mitochondrial function has a critical role during the diauxic shift of yeast.

Yeast has two energy-generating status: fermentation and respiration. In industrial fermentation processes, yeast cells undergo transitions between the two metabolic status. For example, transferring yeast cells from a starter culture, which is often incubated with aeration, to a batch culture would accompany the transition from respiration to fermentation. Although it has been shown that yeast cells retain their mitochondria during alcoholic fermentation, the role of mitochondrial function(s) during such metabolic transitions has not been studied. In order to examine whether yeast mitochondria play unknown roles in the transition, I investigated the role of mitochondrial function during the diauxic shift from fermentation to respiration.

The genome-wide expression of genes was compared be-

tween respiration-competent WT cells and respiration-defective petite cells, in which mitochondrial DNA was depleted. As a result, it turned out that the respiration-defective petite cells were defective in upregulation of certain genes across the diauxic shift (Fig. 1). The genes that failed to be upregulated during the diauxic shift in the respiration-defective petite cells did not exactly correspond to the glucose-repressed genes, indicating that mitochondrial function specifically regulates diauxic-associated genes.

Taken together, our results demonstrated for the first time that mitochondrial function(s) is necessary for the diauxic shift²⁾.

2. Breeding of a mitochondrial morphology-targeted brewery yeast.

I have previously demonstrated that mitochondrial structures of yeast undergo fragmentation during sake brewing (Fig. 2)¹⁾.

Based on this finding, I aimed to breed a novel brewery yeast. I came up with an idea of altering the flux of substances within yeast cells by altering the structure of mitochondria during sake brewing. To achieve this goal, I first tried to determine the factor responsible for fragmentation of mitochondria during sake brewing. I first investigated which factor is responsible for mitochondrial fragmentation

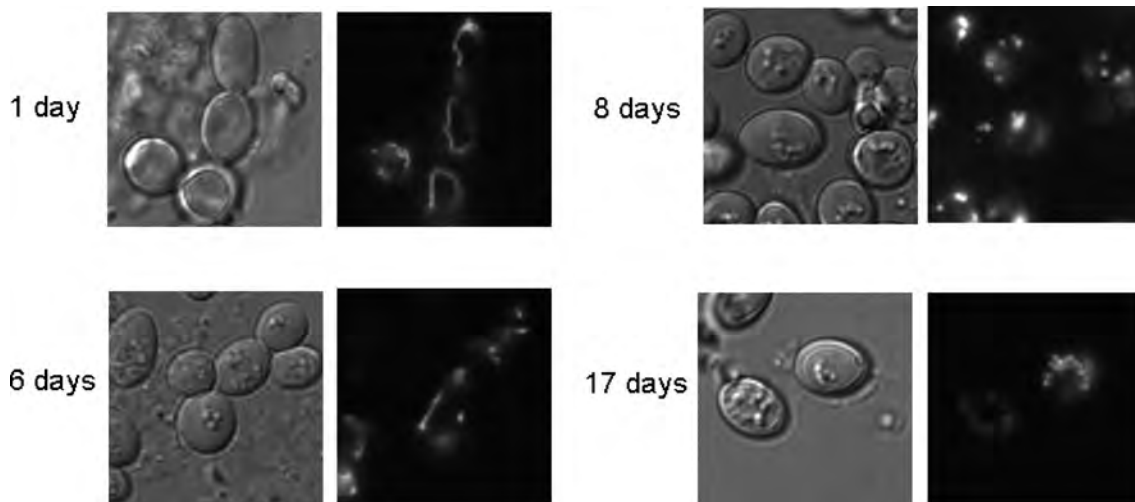


Fig. 2. Mitochondrial structure of wild type sake yeast during sake brewing.

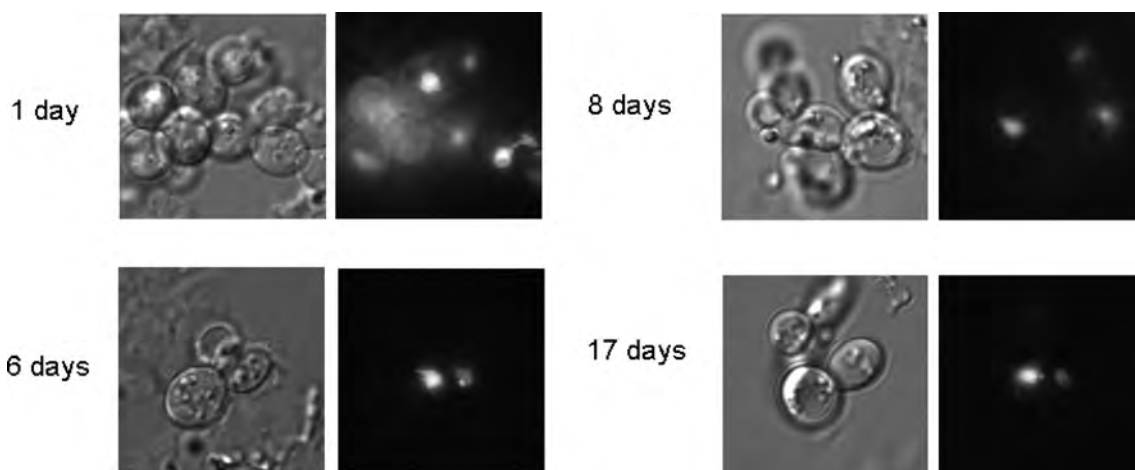


Fig. 3. Mitochondrial structure of *fis1*-disrupted sake yeast during sake brewing.

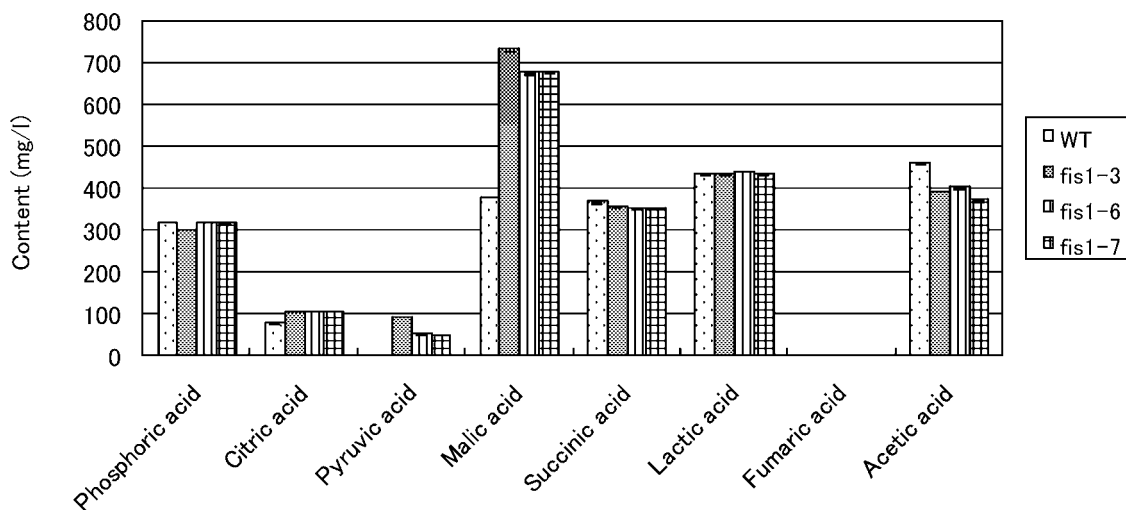


Fig. 4. Organic acid content of sake brewed with the parent strain and *fis1* disruptants of sake yeast.

upon ethanol treatment of yeast cells. It turned out that *FIS1* is responsible for mitochondrial fragmentation upon ethanol treatment³). *Fis1* has been reported to be the factor responsible for mitochondrial fragmentation during vegetative growth⁴). Thus, I disrupted *FIS1* gene in the sake yeast background. I found that the *fis1* disruptant obtained has a networked mitochondrial structure.

I then brewed sake with this *fis1* disruptant, and found that mitochondria of this strain keep the networked structure throughout sake brewing, indicating that *Fis1* is responsible for mitochondrial fragmentation during sake brewing.

As a result of these experiments, I obtained a sake yeast strain with networked mitochondrial structure. I brewed sake with this strain, and measured the organic content of sake. It turned out that the amount of malate, which has a crispy taste, is increased by two folds. This result clearly indicates that mitochondrial structure in yeast cells during sake brewing has a critical role in determining organic acid content of sake⁵⁻¹⁰).

Conclusion

I have demonstrated that (1) mitochondrial function has a critical role during the diauxic shift, and (2) mitochondrial structure can be a target of breeding of brewery yeast strains.

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