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Inhibition of Mitochondrial Fragmentation during Sake Brewing Causes High Malate Production in Sake Yeast

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We previously demonstrated the presence and fragmentation of mitochondria during alcohol fermentation. Here, we show that Fis1p induces mitochondrial fragmentation, and inhibition of mitochondrial fragmentation causes higher malate production during sake brewing. These findings indicate that mitochondrial morphology affects the metabolism of constituents, providing a breeding strategy for high-malate-producing yeasts.

[**Key words:** malate, alcohol fermentation, sake, yeast, mitochondria, Fis1p]

The mitochondrion is an important organelle in eukaryotic cells and is considered to have been acquired through endosymbiosis. Although mitochondria are involved in many aspects of cell function, such as respiration, metabolism of lipids (1) and amino acids (2), storage of metal ions (3), and apoptosis (4), the presence, structure, and role of mitochondria during alcohol fermentation had not been investigated until our previous study (5), perhaps because mitochondrial genes in yeast are repressed by a high concentration of glucose (6) or a low oxygen concentration (7). In our previous study (5), we demonstrated the presence of mitochondria during alcohol fermentation for the first time and that their structures fragment during the brewing of one type of alcoholic beverage, sake, or Japanese rice wine, perhaps owing to the high concentration of ethanol (4). These findings suggest that mitochondria play an important role in the metabolism of constituents produced during sake brewing.

Sake is a traditional Japanese alcoholic beverage produced by saccharifying steamed rice in the presence of *Aspergillus oryzae* and by converting glucose to ethanol using the yeast *Saccharomyces cerevisiae*. Attempts to increase the variety of aromas and tastes of sake have recently been made by many researchers in an effort to stimulate consumers' attention towards sake (8–17). Organic acids contribute significantly to the overall taste of sake. For example, malate has a crispy and refreshing taste (18). Therefore, increasing the content of malate has been considered as a promising approach to increasing the variety of sake.

In this study, we identify the factor involved in mitochondrial fragmentation during sake brewing, and determine that inhibition of mitochondrial fragmentation of industrial sake yeast strains during sake brewing leads to higher production of malate in sake. This is the first study demonstrating that mitochondrial morphology plays a role in the metabolism of constituents during alcohol fermentation and providing a novel strategy for breeding industrial yeast strains that produce higher concentrations of malate.

The *Saccharomyces cerevisiae* strain BY4743 (*MAT* **a**/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*) and *fis1*Δ having the BY4743 background were purchased from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). The sake yeast Kyokai no. 7 (K7) was purchased from the Brewing Society of Japan (Tokyo). The K7 haploid strain K7H868 was obtained by sporulating the K7 parental diploid strain and was selected on the basis of a brewing performance similar to that of the K7 parental diploid strain. Yeast cells were incubated in 2% bactopeptone, 1% bacto-yeast extract (Beckton Dickinson, San Jose, CA, USA) and 2% glucose at 30°C with shaking. Cells harboring mitochondria-targeted GFP (5) 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 appropriate amino acids (MP Biomedicals, Aurora, OH, USA), and 2% glucose at 30°C with shaking. Sake brewing, determination of ethanol concentration, and microscopy observation were performed as described previously (5). The content of organic acids was determined by high-performance liquid chromatography (HPLC) using model LC-10AD (Shimadzu, Kyoto) equipped with a conductivity detector (model CDD-10A; Shimadzu) and a Shim-pack SPR-H column (Shimadzu). Aliquots (10 μl) of samples were applied to the column using an automatic sampler SIL-10AD (Shimadzu) and eluted with 4 mM *p*-toluenesulfonic acid at a flow rate of 0.8 ml/min. The column oven temperature was 40°C. The *FIS1* or *LEU2* gene in the K7H868 strain was disrupted by transformation (19) using a disruption cassette amplified with primers fis1natfw (AAAAACGGCACATAGAAGCACAGATCAGAGCACAGCCATACAACATA

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Abbreviations: DIC, differential interference contrast; GFP, green fluorescent protein; K7, Kyokai no. 7; mito-GFP, mitochondria-targeted GFP; SEM, standard error of mean.

AGTCACATACGATTTAGGTGACAC) and fis1natrv (TGCGATTCATTCTTATGTATGTACGTATGTGCTGATTTTTTATGTGCTTGAATACGACTCACTATAGGGAG), or leu2kanMXfw (TTTACATTTCAGCAATATATATATATATATTTCAAGGATATACCATTCTACAGCTGAAGCTTCGTACGC) and leu2kanMXrv (ATTTCATTTATAAAGTTTATGTACAAATATCATAAAAAAAGAGAATCTTTGCATAGGCCACTAGTGGATCTG), respectively. The transformants were incubated and selected on plates containing 100 μg/ml nourseothricin or 500 μg/ml G418, respectively. Disruption of the *FIS1* gene was confirmed by PCR analysis and staining with mitochondrial dye (50 nM rhodamine 123, Invitrogen, Grand Island, NY, USA).

In our previous study, we elucidated for the first time that yeast mitochondria fragment during alcohol fermentation (5) but not the mechanism of mitochondrial fragmentation during sake brewing. Therefore, here, we first attempted to identify the factor involved in mitochondrial fragmentation during alcohol fermentation. A mitochondrial fission protein, Fis1p, has been reported to be responsible for mitochondrial fragmentation during vegetative growth (20) and acute ethanol stress (4) in yeast. Therefore, we investigated whether disruption of the *FIS1* gene leads to inhibition of mitochondrial fragmentation during alcohol fermentation. Sake was brewed for 22 d with either WT or *fis1*Δ of a laboratory strain (BY4743) harboring mitochondria-targeted GFP (21, 22), and mitochondrial morphology was visualized by fluorescence microscopy. Sake brewing proceeded without any observable defects, as verified by the final decrease in the weight of the mash (WT: 32.23 g, *fis1*Δ: 33.83 g). Although WT mitochondria fragmented as the brewing proceeded (Fig. 1A), as we reported previously (5), *fis1*Δ mitochondria remained networked until the end of brewing (Fig. 1A). This result clearly indicates that Fis1p is responsible for mitochondrial fragmentation during sake brewing.

Next, we investigated the effect of disrupting the *FIS1* gene on the mitochondrial morphology in an industrial sake yeast strain. Mitochondria were observed to transform from having a tubular morphology to a dotted morphology during sake brewing in an industrial strain (K7 [RAK1536] (23) + pRS413GPD-mito-GFP) (21, 24), similar to the transformation observed in the laboratory strain (Fig. 1B). Since the industrial sake yeast strain is a diploid and disruption of the *FIS1* gene requires two steps, a haploid strain (K7H868) having a brewing performance similar to that of the parental diploid strain was obtained by sporulation, and the *FIS1* gene was disrupted in K7H868. The mitochondrial morphology of the haploid *fis1*Δ strain of sake yeast (K7 haploid *fis1*::natMX4 *leu2::kanMX4* + pYX142-mito-GFP) remained networked throughout sake brewing as observed in the laboratory *fis1*Δ strain (Fig. 1B). These findings clearly indicate that Fis1p is also responsible for this fragmentation in the industrial strains.

Since the above results indicated that disruption of the *FIS1* gene results in inhibition of mitochondrial fragmentation during sake brewing, we next investigated the effect of inhibiting mitochondrial fragmentation during sake brewing on the metabolism of constituents during sake brewing by disrupting the *FIS1* gene. Organic acids are important constituents of sake, and several studies suggest the partial involvement of mitochondria in the formation of organic acids during sake brewing (11, 12). Therefore, we analyzed the effect of the disruption of the *FIS1* gene on the formation of organic acids during sake brewing. Sake was brewed with WT and *fis1*Δ strains of a laboratory strain, BY4743, for 22 d, and the contents of organic acids were analyzed and compared. Sake brewing proceeded without significant deviation, as revealed by the amount of carbon dioxide evolved (Fig. 2A) with normal final ethanol concentrations (WT: 15.6%, *fis1*Δ: 16.3%). The analysis showed that the content of malate, an important organic acid, increased in *fis1*Δ

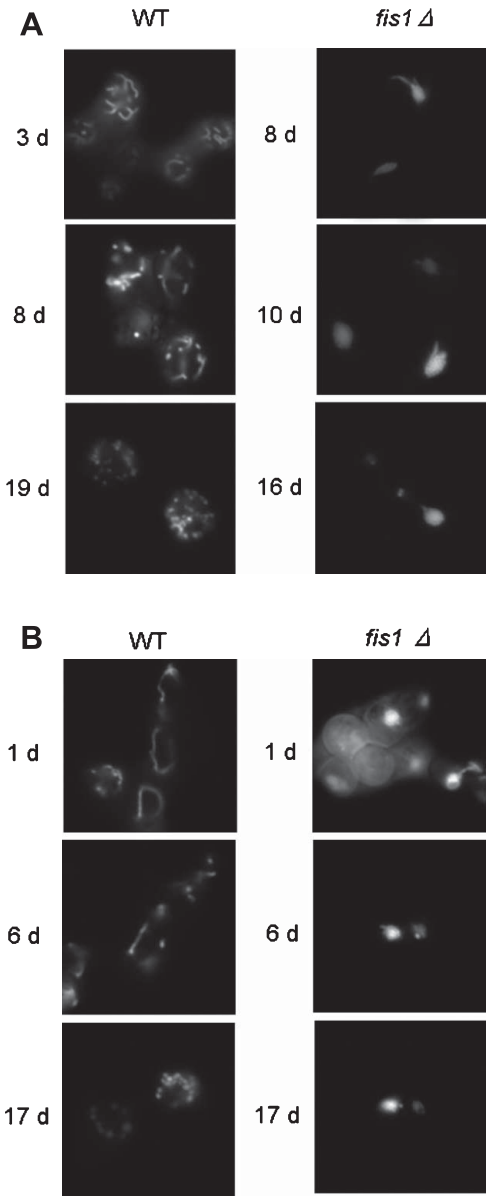

FIG. 1. Mitochondrial morphology of WT and *fis1*Δ obtained from laboratory and industrial strains during sake brewing. Sake was brewed using WT and *fis1*Δ cells expressing mitochondria-targeted GFP, as described in the text. A laboratory strain (BY4743) (A) and industrial strains (Kyokai no. 7 strain [WT] and K7H868 strain [*fis1*Δ]) (B) were used as parental strains. An aliquot was sampled from the mash and GFP signal was directly observed under a fluorescence microscope within a few minutes.