Enhancement of the Initial Rate of Ethanol Fermentation Due to Dysfunction of Yeast Stress Response Components Msn2p and/or Msn4p†

Daisuke Watanabe, Hong Wu, Chiemi Noguchi, Yan Zhou, Takeshi Akao, and Hitoshi Shimoi*

National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan

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Sake yeasts (strains of Saccharomyces cerevisiae) produce high concentrations of ethanol in sake fermentation. To investigate the molecular mechanisms underlying this brewing property, we compared gene expression of sake and laboratory yeasts in sake mash. DNA microarray and reporter gene analyses revealed defects of sake yeasts in environmental stress responses mediated by transcription factors Msn2p and/or Msn4p (Msns2/4p) and stress response elements (STRE). Furthermore, we found that dysfunction of MSN2 and/or MSN4 contributes to the higher initial rate of ethanol fermentation in both sake and laboratory yeasts. These results provide novel insights into yeast stress responses as major impediments of effective ethanol fermentation.

During sake brewing, rice starch is saccharified by enzymes produced by koji mold (Aspergillus oryzae), and the resultant glucose is fermented by sake yeast, which produces ethanol to concentrations reaching approximately 20% (vol/vol), the highest level among nondistilled alcoholic beverages. Sake fermentation produces much more ethanol than laboratory strains of S. cerevisiae in sake mash. One of the reasons for the high ethanol production of sake yeast is its high fermentation rate (28). This property is a critical prerequisite for sake yeast strains because rapid and high-level ethanol accumulation leads to shortening fermentation periods, as well as preventing growth of unwanted microorganisms during sake brewing, in which open fermentation tanks are usually used. Therefore, yeast strains with higher fermentation rates have historically been selected as sake yeasts. In particular, modern sake yeast strains isolated during the last 80 years (also referred to as the “K7 group” [3]), represented by the most popular sake yeast, Kyokai no. 7 (K7), show high fermentation rates and are superior in flavor and aroma production. To explore the molecular mechanisms responsible for the high fermentation rate of modern sake yeast, we previously performed DNA microarray analyses of sake yeast strain Kyokai no. 701 (K701) (genetically almost identical to K7, but with a nonfoaming phenotype [23]) in fermenting sake mash (29). In this study, we compared these microarray data with data obtained from an experiment involving the laboratory yeast strain X2180 under identical fermentation conditions in an attempt to identify the underlying factors affecting ethanol production by sake yeast.

Yeasts stress responses have been considered to be keys to the improvement of ethanol fermentation. A number of recent studies on industrial bioethanol production focus on modification of ethanol productivity through intensification of stress response machineries (20, 30). Msns2p and Msns4p (Msns2/4p) are functionally redundant transcription factors that have been the best characterized among regulators of environmental stress responses (11, 15, 21, 25). In response to various stresses, these factors migrate into the nucleus and bind to stress response elements (STRE) (CCCTT or AGGGG) within the promoters of stress-induced genes. The zinc finger DNA binding domains of Msns2/4p located at their C termini are essential for recognition of STRE sequences (21, 25). Interaction of Msns2p and STRE leads to global transcriptional activation of a large set of stress-responsive genes (8, 13), including those related to oxidative stress defense (e.g., CTT1 and SOD2) (5, 15, 21, 25), carbohydrate metabolism (e.g., TPS1, TPS2, GSY2, ALD2, ALD3, and TKL2) (5, 10, 17, 22, 25), and protein folding chaperones (e.g., HSP12, HSP104, SSA4, and SSA4) (1, 5, 15, 17, 21, 25). Consistently, a Δmsn2 Δmsn4 double mutant displays severe defects in stress-protective gene expression and thus exhibits pleiotropic stress sensitivity (11, 21). Here, we report an unexpected finding that Msn2p-mediated stress responses decrease the initial rate of ethanol fermentation during sake brewing.

MATERIALS AND METHODS

Strains and plasmids. Sake yeast strains Kyokai no. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 (K1, K2, K3, K4, K5, K6, K7, K8, K9, K10, K11, K12, K13, K14, and K15, respectively) and K701 (a nonfoaming variant of K7) were provided by the Brewing Society of Japan. A sake yeast strain (Yabe Kozai), shochu strains (S-2 and SH-4), wine strains (EC-1118, Montrachet, and OC-2), and other alcoholic strains (Bu9-7, S-3, RIB1023, RIB1024, and 389) were provided by the National Research Institute of Brewing (Japan). Beer strains (NCYC120, NCYC1026, NCYC242, and NCYC994) were provided by the National Collection of Yeast Cultures (United Kingdom). Laboratory strains X2180, X2180-1A, Σ1278b, A364A, and W303-1A were provided by the American Type Culture Collection. The BY4743, BY4743 Δmsn2, and BY4743 Δmsn4 strains were provided by EUROSCARF (Germany).

Disruption of the MSN2 or MSN4 gene in X2180-1A was performed by a PCR-based method (14) with primers MSN2-D1 (5'-CTTTTTITTTTGGTTTTATTT GCCTATTTTTCTTTCTTTTCTTTTAACTTCTGTCATAGAAGAACTA GATCTAAGAACTGCGCCGAGCGAC-3') and MSN2-D2 (5'-ATAAGGCCG TAAGGTTATAAAGGCGAAGAATTAATTTCTTTCTTTAGAAGAAGACTA TCGAATTTAAAAAATGGGTTTTAATCGATGAATTCGAGCTCG-3') (26)

† Corresponding author. Mailing address: National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan. Phone: 81-82-420-0825; Fax: 81-82-420-0809. E-mail: simo@nrib.go.jp.

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or MSN4-DF (5′-ATCAGTGGCCTTTTCCTTTTCTTCTTATAAAAA CAAATATACGTAGCGGAGTGCCAG-3′) and MSN4-DR (5′-TACCGTAG CTGGTGTGGTTATATGCTTTTACCTTTTATTCTGATGAGATTCG A-3′) or MSN4-3F (5′-CGCCCTTTATCAGTTCGGCTTT-3′) and MSN4-3R (5′-CACCGTATCTCAATAGAATCCT-3′). The STRE-pCYC1-lacZ reporter gene was normalized by the protein levels quantified using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). For the reporter gene assay of yeast cells under ethanol stress, yeast cells were precultured overnight in YPD, further cultivated in YPD at 25°C until log phase, and transferred into fresh YPD or YPD containing 8% ethanol (EIOH) for 2 h. β-Galactosidase assays were performed as previously reported (26). For the complementation test of X2180-1A Δmsn4Δ by introducing the pYCI40-ScMSN4 or pYCI40-K7MSN4 plasmid, yeast cells were precultured overnight in YPD containing 300 μl cold distilled water and 400 μl 2× Z buffer and then assayed for β-galactosidase activity, as previously described (2). The expression levels of the lacZ reporter gene were normalized by the protein levels quantified using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). For the reporter gene assay of yeast cells isolated from sake mash made with K701 and X2180 yeast cells, cDNA (0.2 μg) was synthesized from 300 ng of total RNA in a final volume of 20 μl using a PrimeScript RT reagent Kit (Perfect Real Time; TaKaRa). Gene-specific quantitative real-time PCR primers were designed with Primer3 (24) as shown in Table S4 in the supplemental material. cDNA (0.2 μg) was synthesized from 300 ng of total RNA in a final volume of 20 μl using a PrimeScript RT reagent Kit (Perfect Real Time; TaKaRa). qRT-PCR was performed with a LightCycler (Roche), and the expression data were processed by the second-derivative maximum method of LightCycler Software version 3.5. Delta cycle threshold (ΔCT) values were calculated by subtracting the CT of the ACTI gene from the CT of the genes of interest. ΔΔCT values were calculated by subtracting the ΔCT of the X2180-1A sample from the ΔCT of the Δmsn2ΔΔms4Δ and K701 samples. Fold changes were calculated using the 2−ΔΔCT method (19).

**RESULTS**

Sake yeast is defective in Msn2/4p-mediated gene expression in sake fermentation. To identify differences between the sake and laboratory yeast strains that contribute to the superior brewing properties of sake yeast, we compared the gene expression profiles of both types of yeast in sake mash (the fermentation profiles are shown in Fig. S1C in the supplemental material). Analysis of differential DNA microarray data (see Table S3 in the supplemental material) using T-profiler, which is used for scoring changes in the average expression levels of predefined groups of genes (4), revealed that the
expression of genes under the control of several transcription factors that are responsible for stress responses and nutrient signaling, including Msn2/4p, Skn7p, Gcr1p, Put3p, Gcn4p, and Adr1p, was significantly lower in K701 (Table 1). This result suggests that a relationship exists between their dysfunction and the superior brewing properties of sake yeast. Comparing microarray data for K701 and X2180 at similar ethanol levels also gave the same result: K701 exhibited impaired functions of transcription factors involved in stress responses and nutrient signaling (see Table S5 in the supplemental material).

Among the identified transcription factors, we focused on Msn2/4p, as they are known to be crucial for yeast stress responses and their targets were the most remarkably downregulated in the K701 sake mash.

Our DNA microarray results indicate that the induction of known target genes of Msn2/4p, which peaked on day 3 or 5 in X2180, was significantly decreased or nearly completely abolished in the sake yeast (Fig. 1A), consistent with the T-profiler analyses. To confirm these results, we performed qRT-PCR experiments using the samples from the sake mash on day 5.
All the target genes examined showed significantly lower mRNA expression levels in K701 than in X2180-1A (ranging from 4.1% to 77.1%), in agreement with our DNA microarray analyses. Comparison between the expression levels of the X2180-1A wild type and the Δmsn2 Δmsn4 mutant (ranging from 0.8% to 51.3%) verified that the upregulation of the target genes in the sake mash was at least partly dependent on Msn2/4p. These results revealed that Msn2/4p-mediated induction in the sake mash was significantly impaired in K701.

Moreover, we examined the expression of the STRE-pCYC1-lacZ fusion gene in both K701 and X2180 during sake brewing (Fig. 2A). Whereas X2180 exhibited upregula-
tion of the fusion gene from day 3, which indicates that the increasing ethanol concentration was sufficiently stressful to induce activation of Msn2/4p, no significant upregulation was observed in K701. In contrast, K701 exhibited an obvious induction of STRE-driven genes, the same as X2180, under the acute 8% ethanol stress in YPD medium (Fig. 2B). Altogether, these data demonstrate that sake yeast has severe defects in stress-inducible gene expression mediated by Msn2/4p and STRE specifically during sake brewing.

Loss-of-function mutations in the MSN4 gene are specifically distributed in modern sake yeast strains. To investigate the cause of the defective stress responses in sake yeast, we focused on nucleotide polymorphisms in the MSN2 and MSN4 genes (Fig. 3A). MSN2 nucleotide sequences are well conserved between K7 and X2180, except for 3 nonsynonymous polymorphisms. MSN4 has more nonsynonymous polymorphisms. We previously identified two point mutations (T2C and C1540T) in sake yeast K7 that result in the deletion of the N and C termini, respectively, of Msn4p (26). Of these mutations, C1540T is considered a loss-of-function mutation, as it deletes the C-terminal zinc finger DNA binding motifs of Msn4p. We therefore examined the functionality of Msn4p using the X2180-1A Δmsn4 strain complemented with either functional S. cerevisiae MSN4 (ScMSN4) or double-truncated MSN4 from K7 (K7MSN4). Upon heat shock, Δmsn4 showed a significant decrease of STRE-pCYC1-lacZ fusion expression compared to wild-type cells. Although the introduction of ScMSN4 rescued this impaired induction in the Δmsn4 strain, K7MSN4 did not contribute to the STRE-dependent stress response (Fig. 3B). We can therefore conclude that K7Msn4p no longer functions as a stress-responsive transcription factor.

Next, we examined the distribution of the T2C and C1540T polymorphisms among 17 sake, 3 wine, 4 beer, 2 shochu, 5 other alcohol, and 5 laboratory yeast strains (Fig. 3C). This comparison revealed that genetically closely related modern sake yeast strains, including Kyokai no. 6, 9, 10, 11, 12, 13, 14, and 15 (3), had mutations identical to those of K7 and K701. In contrast, nearly all of the other yeast strains tested, including the classical sake yeast strains (Kyokai no. 1, 2, 3, 4, 5, and 8 and Yabe Kozai), as well as the wine, beer, and laboratory yeast strains, showed no double truncation of MSN4, as was observed for X2180. Furthermore, fungal sequence alignment analysis of sequences in the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/) revealed that both the T2 and C1540 nucleotides are universally conserved among orthologous genes in Saccharomyces sensu stricto. These data suggest that an ancestral strain of the modern sake yeasts may have acquired loss-of-function mutations in the MSN4 gene during the selection of sake yeast with desirable brewing properties.

Expression of the functional MSN4 gene delays ethanol fermentation in sake yeast. To analyze the relationships between defective Msn4p and the brewing properties of the sake yeast, we performed small-scale sake-brewing tests (Fig. 4A and B). Expression of the functional MSN4 gene from a low-copy-number plasmid in K7 (K7/ScMSN4) led to delayed fermentation in the early stages of sake brewing (Fig. 4A). From days 2 to 5, carbon dioxide generation by the K7 + ScMSN4 strain...
was significantly lower than that of the control strain (Fig. 4B). In addition, we examined fermentation ability in the presence of 20% glucose utilizing Fermograph II, an automated gas-monitoring instrument (D. Watanabe, T. Akao, and H. Shimoi, submitted for publication), which revealed that the K7 + ScMSN4 strain also displayed slower fermentation than the control under this condition (Fig. 4C and D). Taken together, these results demonstrate that expression of functional Msn4p negatively acts on the ethanol fermentation rate of K7 and that dysfunction of Msn4p at least partly contributes to the high ethanol fermentation rate of K7.

**Loss of MSN2 and/or MSN4 enhances the initial rate of ethanol fermentation.** Based on the fermentation test results described above, we hypothesized that the loss of Msn2/4p functions might lead to improved ethanol fermentation in general yeast strains other than sake yeast. We thus performed fermentation tests using the Δmsn2, Δmsn4, and Δmsn2 Δmsn4 disruptants in a laboratory yeast background (X2180-1A; haploid) (Fig. 5). It was revealed that all three disruptant strains displayed significant increases of evolved carbon dioxide gas in the early stages of sake brewing compared to the wild-type strain, with the double mutant showing the largest increase (Fig. 5A to D). After 20 days of fermentation, the ethanol concentrations were higher and the specific gravities were lower in sake produced using the disruptants (Table 2), also indicating that the abrogation of Msn2/4p leads to improved fermentation. Since the other examined brewing characteristics were not remarkably altered in the disruptants (Table 2), the major effects caused by loss of Msn2/4p functions are considered to be improvement of the initial fermentation rate and gross ethanol production. Furthermore, the disruptants also exhibited more rapid fermentation in 20% glucose-containing YPD medium at 30°C than the wild-type strain (Fig. 5E and G). In addition, we confirmed that the deletion of MSN2 or MSN4 in another genetic background (BY4743; diploid) gave results similar to those in the X2180-1A background (Fig. 5F and H). From these results, we demonstrated that impairment of Msn2/4p functions improves fermentation efficiency in various yeast strains regardless of the genetic background or the ploidy.

**DISCUSSION**

Yeast stress responses and the resultant stress tolerance are generally considered to be important characteristics for effective ethanol fermentation; therefore, numerous recent studies have focused on the enhancement of ethanol tolerance to achieve high ethanol productivity (20, 27, 30). Contrary to this approach, however, our present experimental data reveal that Msn2/4p-mediated environmental stress responses act as a physiological “brake” for ethanol production in both sake and laboratory yeast strains. The yeast strains defective in Msn2/4p exhibited significant increases of the peak fermentation rate in the early stages, leading to “powerful fermentation,” a novel concept aimed at advancing brewing technologies, such as optimization of continuous-culture methods for industrial bio-ethanol production.

Concerning the underlying molecular mechanisms that drive powerful fermentation, we hypothesize that minimal stress responses might contribute to facilitating the initial fermentation
rate. In the early stages of fermentation, yeast cells might not need to rapidly acquire stress tolerance because the ethanol concentration is not increasing acutely, nor has it reached toxic levels. Therefore, the integrity of stress-responsive machineries might be less significant in this period. In fact, several stress response transcription factors, including Msn2/4p, of sake yeast were actually inactivated in the initial stage of fermentation, as shown in Table 1. Thus, yeast stress responses might be dispensable (or rather, inhibitory) in the early stage of ethanol fermentation. This idea is also in agreement with the evidence for impaired expression of heat shock proteins in beer and wine yeast strains in practical use (6, 7) and for high ethanol production by disruption of stress-responsive ubiquitin-related genes (28).

Moreover, it is noteworthy that yeast stress response machineries negatively affect cell growth, although they are important for maintaining cellular viability under acute stresses. For instance, activation of Msn2p or its overexpression under the control of a constitutive promoter has detrimental effects on cell growth and proliferation (9, 21). Considering the costs of stress responses, yeast strains with reduced stress-responsive gene expression might have advantages in increasing the population size under early fermentation conditions with moderate stress. In addition, yeast stress responses often result in drastic changes to cellular metabolic states, which might also affect powerful fermentation. The environmental stress responses mediated by Msn2/4p activate the expression of several genes involved in carbohydrate assimilation, such as trehalose and glycolysis metabolism, pentose phosphate shuttling, fatty acid metabolism, and respiration (12), which also supports the idea that excess stress responses might have inhibitory effects on the glycolysis and ethanol production efficiency of individual cells by diversifying carbon flux. Thus, the stress response components of industrial yeast strains likely have been fine-tuned by natural selection.

How are the Msn2/4p-mediated stress responses of sake yeast inactivated during sake fermentation? We found that the modern sake yeasts, including K7, had nonfunctional MSN4 alleles (Fig. 3), and expression of functional MSN4 in K7 led to decrease of the initial fermentation rate (Fig. 4), suggesting that dysfunction of Msn4p might partly contribute to the powerful fermentation of K7. We speculate that an ancestral strain of the modern sake yeasts might have lost the functions of Msn4p during the artificial selection of sake yeast with a higher fermentation rate. However, this alteration in MSN4 is not sufficient to entirely explain the powerful fermentation of K7. Msn2p is redundant with Msn4p and is largely responsible for STRE-mediated transcription (25). In our data, STRE-driven gene expression was not significantly induced during sake fermentation by K701 (Fig. 2A) but was strongly induced in K701 under acute ethanol stress, almost the same as in X2180 (Fig. 2B). These results suggest that Msn2p of K701 is functional enough to induce STRE-pCYC1-lacZ under acute ethanol stress but is not activated by chronic stresses existing in sake mash (e.g., a gradual increase of the ethanol concentration).

To understand the molecular basis for this phenomenon, we assume that an upstream activator(s) of Msn2/4p that specifically responds to chronic stresses under sake-brewing conditions is completely defective in K701 (and also in other sake yeast strains). More detailed analysis of the loss-of-function mutations in the sake yeast genome would reveal the central molecular mechanism responsible for both inactivation of Msn2/4p under chronic stresses and the powerful fermentation characteristic of sake yeast.

We also note that the defects in stress response components might cause some negative consequences, for example, difficulty in long-term survival in the stressful environment due to defective stress tolerance. In fact, most K701 cells lost their viability after sake fermentation was finished (see Fig. S1 in the supplemental material). This phenotype has not been a serious problem in the selection of sake yeast strains, however, since sake mash is filtered soon after the end of fermentation before yeast cell death, and recovered yeast cells are not reused for the next round of sake brewing.

We previously reported the increase of the final ethanol concentration in the sake mash fermented by a sake yeast strain overexpressing MSN2 (27). Reduced stress response and yeast cell death of sake yeast at the end of sake brewing may explain the apparent discrepancy that both inactivation and activation of Msn2/4p result in greater fermentation ability. While the excess level of MSN2 does not have an obvious effect on the initial rate of sake fermentation, it significantly improves the fermentation rate in the latter half of sake brewing; this is probably because stress tolerance caused by Msn2p might act positively on maintaining cell viability under severe ethanol stress. In contrast, the powerful fermentation pheno-
type attributed to loss of MSN2 and/or MSN4 is defined by enhancement of the initial fermentation rate. As shown in Fig. 5B and C, only the peak fermentation rates are significantly improved in the Δmsn2 and/or Δmsn4 disruptants. In addition, the fermentation rate in the latter stage is significantly reduced in the Δmsn2 Δmsn4 double mutant (Fig. 5D), suggesting that this strain might be too sensitive to ethanol stress to sustain its fermentation rate under the elevated ethanol concentration. We thus speculate that Msn2/4p-mediated stress responses contribute negatively to the fermentation rate in the initial stage and positively in the latter stage of the brewing process.

In conclusion, our results suggest that the inactivation of Msn2/4p may play a role in the high initial fermentation rate of modern sake yeast strains compared to other natural and industrial yeast strains. We suggest that stress responses have not evolved to promote rapid metabolism but to protect the organism from adverse conditions and to limit its tendency to contribute negatively to the fermentation rate in the initial stage and positively in the latter stage of the brewing process.

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