Accelerated Alcoholic Fermentation Caused by Defective Gene Expression Related to Glucose Derepression in *Saccharomyces cerevisiae*

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Sake yeast strains maintain high fermentation rates, even after the stationary growth phase begins. To determine the molecular mechanisms underlying this advantageous brewing property, we compared the gene expression profiles of sake and laboratory yeast strains of *Saccharomyces cerevisiae* during the stationary growth phase. DNA microarray analysis revealed that the sake yeast strain examined had defects in expression of the genes related to glucose derepression mediated by transcription factors Adr1p and Cat8p. Furthermore, deletion of the *ADR1* and *CAT8* genes slightly but statistically significantly improved the fermentation rate of a laboratory yeast strain. We also identified two loss-of-function mutations in the *ADR1* gene of existing sake yeast strains. Taken together, these results indicate that the gene expression program associated with glucose derepression for yeast acts as an impediment to effective alcoholic fermentation under glucose-rich fermentative conditions.

Key words: *ADR1*; alcoholic fermentation; glucose derepression; *Saccharomyces cerevisiae*; sake yeast

Sake yeast strains, which belong to the budding yeast species *Saccharomyces cerevisiae*, produce markedly more ethanol during sake fermentation than any other *S. cerevisiae* strains, including common laboratory ones. Since a rapid accumulation of high concentrations of ethanol is desirable in sake brewing in order to shorten fermentation periods, yeast strains with higher fermentation rates have been selected historically as sake yeast. Several strains were developed since the discovery of sake yeast in sake mash at the end of nineteenth century,¹ and those strains with outstanding brewing properties have been controlled and administered by the Brewing Society of Japan. The most widely used sake yeast strain, Kyokai no. 7 (K7), originally isolated in 1946,² has since been serially subcultured, as opposed to deep-freezing storage, to preserve its characteristic traits. Thus the genetic variations responsible for the superior brewing properties of existing sake strains may have been preserved through repeated subculture, whereas the functioning of genes that have less biological importance may have been lost.

In previous studies, we identified modern sake yeast-specific loss-of-function mutations in the *RIM15* (*rim15<sup>5055insA</sup>*<sup>3</sup>) and *MSN4* (*msn4<sup>C1540T</sup>*)<sup>4</sup> genes, both of which are involved in entry into quiescence during the stationary phase of cell growth. Under stress conditions, yeast Per-Arnt-Sim kinase (PASK) Rim15p activates a quiescent gene expression program mediated by redundant stress-responsive transcription factors Msn2p and Msn4p (*Msn2/4*)<sup>5,6</sup>. Due to mutations in the *RIM15* and *MSN4* genes, modern sake yeast cells in the stationary phase share common phenotypes, including low thermotolerance, ethanol susceptibility, low buoyant density, decreased production of reserve carbohydrates trehalose and glycogen, and impaired cell-cycle G1 arrest.<sup>3,4,7,8</sup> Furthermore, deletion of these genes significantly enhances the fermentation rates of non-sake yeast strains,<sup>9,3,4</sup> suggesting that the *rim15<sup>5055insA</sup>* and *msn4<sup>C1540T</sup>* mutations play pivotal roles in increasing the fermentation rates of modern sake strains. A question arises here: do these mutations alone account for the high fermentation rates of modern sake yeast strains? In a previous DNA microarray analysis of yeast cells in fermenting sake mash,<sup>9</sup> we found that other several transcription factors, including Gcn4p and Adr1p, are also significantly impaired in sake yeast. These transcription factors might be involved in the negative control of alcoholic fermentation cooperatively with or independently of the Rim15p-Msn2/4p pathway.

Adr1p-mediated release from glucose repression, known as glucose derepression, is one plausible mechanism that might interfere with yeast alcoholic fermentation in the stationary phase. In *S. cerevisiae*, a large set of genes is repressed by glucose, including genes involved in the utilization of alternate carbon sources, including ethanol, glycerol, sucrose, galactose, maltose, and organic acids, and in gluconeogenesis, respiration, and peroxisomal function. In contrast, after the cessation of fermentation, glucose repression is released, and genes related to alternative carbon sources become derepressed. In sake and other types of alcoholic beverages, glucose repression is released when a sufficient amount of sugar or alcohol has accumulated, and the cell enters the stationary growth phase. However, sake yeast cells are capable of fermenting at very low concentrations of glucose (0.02%), and the stationary growth phase is prolonged after the stationary phase of cell growth begins. Under this condition, the activity of the Rim15p-Msn2/4p pathway is considered to be lower than that in non-sake yeast. This mechanism might contribute to the high fermentation rate of modern sake yeast strains.

In this study, we aimed to identify novel transcription factors involved in the negative control of alcoholic fermentation in sake yeast.
of logarithmic growth in response to glucose limitation, these glucose-repressed genes are upregulated. Glucose repression/derepression of these genes enables yeast cells to adapt their metabolism for optimal utilization of carbon sources in dynamic nutrient environments. However, under industrial fermentative conditions, including those for sake mash, in which abundant fermentable sugars are continuously supplied to yeast cells, glucose derepression might be unnecessary for effective glucose utilization and alcoholic fermentation. Especially, utilization of the ethanol produced during fermentation might directly lead to a decrease of ethanol yield.

Yeast AMP-activated protein kinase (AMPK) homolog Mig1p is essential for regulating glucose repression/derepression mediated by transcriptional repressor Mig1p and transcriptional activators Adr1p and Cat8p. The Mig1 gene, which was first isolated as a multicopy inhibitor of galactose gene expression, encodes a zinc-finger DNA-binding protein that represses expression of the genes involved in the utilization of non-glucose sugars. ADR1 was identified as a regulatory gene that controls the expression of the glucose-repressed ADH2 gene, which encodes an alcohol dehydrogenase isozyme required for the first step in ethanol oxidation. Yeast cells were grown in YPD medium (1% yeast extract, 2% bacto-tryptone, 2% glucose) provided by the American Type Culture Collection (Boston, MA). The primer pairs used were as follows: ADH1-RT-F (5’-GACCTTACCGAGTCCG-3’), ADH1-RT-R (5’-TTTTTATTCAGTTATT-3’), and CAT8-Fw (5’-CTATGTTGTCATTGACGCTAC-3’), and CAT8-Rv (5’-CTACTAAG-3’). Gene expression was determined by measuring the amount of evolved carbon dioxide, which was monitored by measuring the weight loss of the sake mash at the same time each day. Measurement of fermentation rate. For fermentation tests in YPD medium at 30 °C with vigorous shaking, Purification of the crude RNA solutions and subsequent DNA microarray experiments were performed as published. The raw and normalized data sets used in T-profiler analysis are shown in Supplemental Tables 1, 2, and 3 (see Biosci. Biotechnol. Biochem. Web site).

Quantitative real-time PCR (qRT-PCR) assay. Yeast cells were grown in YPD medium at 30 °C for 7 d with vigorous shaking. Total RNA was isolated from the cells with an RNeasy Mini Kit (Qiagen, Valencia, CA) and was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized from 50 ng of total RNA in a final volume of 10 μL with a ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan). The PCR mixtures (total volume 20 μL) contained cDNA (0.5 μL for each sample), 0.5 μM primers, and 10 μL of Master Mix 2× conc. of FastStart Essential DNA Green Master Kit (Roche, Mannheim, Germany). The primer pairs used were as follows: ADH1-RT-F (5’-ACCATTTGATACCGGCG-3’), ADH1-RT-R (5’-ACCATTTGATACCGGCG-3’), and ADH2-RT-F (5’-ACCGGCACATCGC-AACTAAG-3’), ADH2-RT-R (5’-ACCGGCACATCGC-AACTAAG-3’), and RIM15-F (5’-CTTTGTTGTCATCAGC-3’), and RIM15-R (5’-TTCTAACAAGGAAATATATACG-3’). Gene-specificqRT-PCR was performed using a LightCycler Nano (Roche) instrument, and the expression data were processed by the automatic quantification mode of LightCycler Nano Software version 1.0 (Roche). Delta cycle threshold (ΔCT) values were calculated by subtracting the CT of the ACT1 gene from the CT of the ADH1 or the ADH2 gene. ΔACT values were calculated by subtracting the ΔCT of the X2180-1A sample from the ΔACT of the X2180-1A Δadr1 Δcat8 or the X2180-1A Δrim15 sample. Fold changes were calculated by the 2−ΔΔCT method.

DNA microarray analysis. Total RNA was extracted by the hot phenol method (K7) from K701 and X2180 cells grown in YPD medium at 30 °C with vigorous shaking. Puriﬁcation of the crude RNA solutions and subsequent DNA microarray experiments were performed as previously described. The raw and normalized data sets used in T-profiler analysis are shown in Supplemental Tables 1, 2, and 3 (see Biosci. Biotechnol. Biochem. Web site).

Materials and Methods

Strains. Sake yeast strains Kyokai no. 7 (K7), its non-foaming variant Kyokai no. 7 (K701), and its ethanol-tolerant variant Kyokai no. 11 (K11) were provided by the Brewing Society of Japan. Laboratory S. cerevisiae strains X2180 and X2180-1A were provided by the American Type Culture Collection (Boston, MA). Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 30 °C with agitation, unless otherwise noted.

Disruption of the ADR1, CAT8, and RIM15 genes in X2180-1A was performed using a polymerase chain reaction (PCR)-based method (K3) with primers ADR1-DF (5’-TTCTGCAGTCTAATTTCTACCTTGCACAGG-3’), ADR1-DR (5’-AAAATAATGCAATAATTTTGTCGAGTTTGAA-3’), CAT8-FF (5’-TTTCTTAGCATTATTCTCCTTAACACAGTGAAGGACCGGCGGCAAC-3’), and CAT8-RR (5’-CTCTACGATAGAATAGATAAGCCCAGTAGAGGAAGACCGTACG-3’). Alternatively, plasmids pAv6-kanMX4 and pAv25 (K20) as templates, to generate mutant yeast strains X2180-1A Δadr1 (K7), X2180-1A Δcat8 (K11), and X2180-1A Δrim15 (K12). These glucose-repressed genes are upregulated. Glucose derepression mediated by transcriptional activator Adr1p and Cat8p is required for the first step in ethanol oxidation.14,15) The Mig1 gene, which was first isolated as a multicopy inhibitor of galactose gene expression, encodes a zinc-finger DNA-binding protein that represses expression of the genes involved in the utilization of non-glucose sugars.10,11) ADR1 was identified as a regulatory gene that controls the expression of the glucose-repressed ADH2 gene, which encodes an alcohol dehydrogenase isozyme required for the first step in ethanol oxidation.14,15) CAT8 was originally identified as a gene necessary for derepression of gluconeogenic enzymes, including fructose-1,6-bisphosphatase (Fbp1p).16) Later, it was also found to be responsible for effective induction of the ADH2 gene.7,10) Although the ADH1 (constitutive alcohol dehydrogenase) and ADH2 (glucose-repressed alcohol dehydrogenase) genes share a high degree of sequence similarity, Adr1p reduces acetaldehyde to ethanol, while Adh2p catalyzes the reverse reaction of oxidizing ethanol to acetaldehyde, due to differences in substrate affinity.10,20) A recent study has provided evidence that a decrease in Adh2p activity might improve the fermentation performance of an industrial brewing strain.21) Furthermore, transcriptomic analyses have revealed that Adr1p and Cat8p act both independently and synergistically to regulate more than 100 genes after diauxic shift.22,23) Several lines of evidence suggest that Adr1p and Cat8p are activated under glucose-rich fermentative conditions,24–26) suggesting a possible novel role of these transcription factors in fermentation control, as well as in glucose derepression under glucose-starved conditions, one that has not been elucidated yet. Here we investigated the relationship between Adr1p/Cat8p-dependent gene expression and alcoholic fermentation in S. cerevisiae.

DNA microarray analysis. Total RNA was extracted by the hot phenol method (K7) from K701 and X2180 cells grown in YPD medium at 30 °C with vigorous shaking. Purification of the crude RNA solutions and subsequent DNA microarray experiments were performed as previously described. The raw and normalized data sets used in T-profiler analysis are shown in Supplemental Tables 1, 2, and 3 (see Biosci. Biotechnol. Biochem. Web site).

Quantitative real-time PCR (qRT-PCR) assay. Yeast cells were grown in YPD medium at 30 °C for 7 d with vigorous shaking. Total RNA was isolated from the cells with an RNeasy Mini Kit (Qiagen, Valencia, CA) and was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized from 50 ng of total RNA in a final volume of 10 μL with a ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan). The PCR mixtures (total volume 20 μL) contained cDNA (0.5 μL for each sample), 0.5 μM primers, and 10 μL of Master Mix 2× conc. of FastStart Essential DNA Green Master Kit (Roche, Mannheim, Germany). The primer pairs used were as follows: ADH1-RT-F (5’-ACCGGCACATCGC-AACTAAG-3’), ADH1-RT-R (5’-ACCGGCACATCGC-AACTAAG-3’), and ADH2-RT-F (5’-ACCGGCACATCGC-AACTAAG-3’), ADH2-RT-R (5’-ACCGGCACATCGC-AACTAAG-3’), and RIM15-F (5’-CTTTGTTGTCATCAGC-3’), and RIM15-R (5’-TTCTAACAAGGAAATATATACG-3’). Gene-specific qRT-PCR was performed using a LightCycler Nano (Roche) instrument, and the expression data were processed by the automatic quantification mode of LightCycler Nano Software version 1.0 (Roche). Delta cycle threshold (ΔCT) values were calculated by subtracting the CT of the ACT1 gene from the CT of the ADH1 or the ADH2 gene. ΔACT values were calculated by subtracting the ΔCT of the X2180-1A sample from the ΔACT of the X2180-1A Δadr1 Δcat8 or the X2180-1A Δrim15 sample. Fold changes were calculated by the 2−ΔΔCT method.

Measurement of fermentation rate. For fermentation tests in YPD medium, yeast cells were precultured in YPD medium overnight at 30 °C, inoculated into YPD medium containing 20% glucose at a final optical density at 660 nm (OD660) of 0.1, and then further incubated at 30 °C without shaking. Fermentation was continuously monitored by measuring the volume of carbon dioxide evolved with a Fermograph II apparatus (Atto, Tokyo, Japan). For the small-scale sake brewing experiments, a single-step sake mash was prepared by mixing 40 g of pre-gelatinized rice, 10 g of dried koji (rice cultivated with Aspergillus oryzae), 20 mL of 90% lactic acid, and 80 mL of water containing yeast cells at a final OD660 of 1.0, and this was then incubated at 15 °C for 20 d without shaking. Fermentation was monitored by measuring the amount of evolved carbon dioxide, which was determined by measuring the weight loss of the sake mash at the same time each day.
Results and Discussion

Sake yeast is defective in Adr1p/Cat8p-dependent gene expression under both aerobic and anaerobic conditions

Considering that alcoholic fermentation by *S. cerevisiae* is gradually attenuated after the cessation of cell growth, a specific cellular physiological change during the stationary phase might be closely related to the observed negative regulation of alcoholic fermentation in this species. To identify differences between sake and laboratory yeast strains that contribute to the stationary phase-specific phenotype of sake yeast, we compared the gene expression profiles of sake yeast strain K701 and laboratory strain X2180 grown aerobically in YPD medium for 7 d. Analysis of differential DNA microarray data after 7 d of culture (Supplemental Table 3) using T-profiler, which is used for scoring changes in the average expression levels of predefined gene groups,32) revealed that expression of genes under the control of several transcription factors, including Cat8p, Mig1p, Adr1p, Swi5p, and Gcn4p, was significantly lower in sake strain K701 (Table 1). None of the gene groups exhibited significantly enhanced expression in K701.

We focused further on the details of the expression profiles of the genes related to glucose derepression, which are downregulated by Mig1p or upregulated by Adr1p and Cat8p. As shown in Fig. 1, the expression of the target genes, which are involved in ethanol utilization (*ADH2, ALD4, ACS1*, and *CIT3*),17,18,22,23,36) acetate utilization (*ADY2 and ACH1*),22,23,37) lactate utilization (*JEN1 and DLD1*),22,23,38,39) glyoxylate cycle (*ICL1* and *MLS1*),22,23,40) and gluconeogenic enzymes (*MDH2* and *FBP1*),18,22,23,41) reached similar levels in the two strains upon diauxic shift (about 1 d). With increasing cultivation time, however, the expression levels of these genes in K701 gradually decreased, whereas those in X2180 remained relatively high, leading to significantly different values at 7 d. The expression levels in K701 at 7 d ranged from 8.0% (*CIT3*) to 59.9% (*DLD1*) of those in X2180. By contrast, the genes not subjected to glucose repression/derepression (*ACT1, ADH1, RPL32*, and *TEF1*) exhibited similar expression levels as between K701 and X2180 throughout the experiment. The expression ratios of K701 to X2180 at 7 d ranged from 85.4% (*ADH1*) to 106.4% (*RPL32*). These data indicate that glucose derepression is specifically impaired in the

### Table 1. T-Profiler Analysis Results for Motifs among Differentially Expressed Genes

<table>
<thead>
<tr>
<th>Motif*</th>
<th>Name</th>
<th>t-value</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCN8N8NCCG</td>
<td>CAT8</td>
<td>−6.63</td>
<td>4.90E−9</td>
</tr>
<tr>
<td>GCGCCCTA</td>
<td>Sporulation</td>
<td>−6.57</td>
<td>7.35E−9</td>
</tr>
<tr>
<td>TSGCCTGCTAWW</td>
<td>Meiosis</td>
<td>−5.54</td>
<td>4.42E−6</td>
</tr>
<tr>
<td>TWCCCCCM</td>
<td>MIG1</td>
<td>−4.49</td>
<td>1.04E−3</td>
</tr>
<tr>
<td>TCTCC</td>
<td>ADR1</td>
<td>−3.80</td>
<td>2.09E−2</td>
</tr>
<tr>
<td>KGCTGR</td>
<td>SWI5</td>
<td>−3.67</td>
<td>3.84E−2</td>
</tr>
<tr>
<td>CGGNNNNNNNNNNKNCGV</td>
<td>Unknown</td>
<td>−3.61</td>
<td>4.37E−2</td>
</tr>
<tr>
<td>GAGTCA</td>
<td>GCN4</td>
<td>−3.58</td>
<td>4.89E−2</td>
</tr>
</tbody>
</table>

*Consensus motifs associated with differentially expressed genes in yeast strains K701 and X2180 grown in YPD medium for 7 d, as determined by DNA microarray data. Only motifs having an E-value of <0.05 are shown.

![Fig. 1. Impaired Expression of the Target Genes of Adr1p and Cat8p in the Stationary Phase of Sake Yeast.](http://www.yeastact.com/)
The extended stationary phase of K701 due to reactivation of Mig1p and/or inactivation of Adr1p and Cat8p. Considering that the common target genes of Mig1p and Adr1p/Cat8p (JEN1, MDH2, and FBP1) and the other Adr1p/Cat8p-target genes exhibited similar profiles (Fig. 1), inactivation of Adr1p and Cat8p is probably a major mechanism causing defective glucose derepression in K701.

To determine whether the observed differences in expression levels were caused by Adr1p and Cat8p, we performed qRT-PCR analysis using X2180-1A and Δ adr1 Δ cat8 disruptant cells of it grown in YPD medium for 7 d (Table 2). As for the results, the expression level of a target gene (ADH2) was severely reduced by deletion of the ADR1 and CAT8 genes, while that of constitutive alcohol dehydrogenase ADH1 was not much affected in the double disruptant. This indicates that the activities of Adr1p and Cat8p were responsible for the elevated expression level of ADH2 in the extended stationary phase.

Based on these results, we suggest that glucose derepression in S. cerevisiae includes at least two separate mechanisms: (i) induction of glucose-repressed genes upon diauxic shift, and (ii) maintenance of glucose-repressed gene expression levels in the extended stationary phase. The former well-characterized process is primarily regulated by Snf1p, which inactivates Mig1p and activates Adr1p and Cat8p, whereas the factors involved in the latter response have yet to be investigated. In sake yeast strain K701, unknown defects in the latter mechanism might have caused the observed decrease in the activities of Adr1p and Cat8p. We postulate that the Rim15p-Msn2/4p pathway, which is specifically inactivated in modern sake strains, is indirectly involved in the maintenance of Adr1p and Cat8p activities. As shown in Table 2, however, deletion of the RIM15 gene did not decrease the ADH2 mRNA level in X2180-1A. Hence we speculate that impaired expression of the genes inducing glucose derepression in the extended stationary phase is a novel feature of sake yeast that is caused independently of defective Rim15p functions.

We have reported that the target genes of Adr1p are also downregulated in K701 in glucose-rich sake mash. Although the roles of Adr1p and Cat8p under hypoxic fermentative conditions are not well established, several lines of evidence suggest that they are activated during fermentation processes. This indicates that Adr1p and Cat8p probably have unknown physiological roles in the control of fermentation even in the presence of abundant glucose, besides their roles as key regulators of glucose derepression under glucose-starved conditions. As shown in Supplemental Fig. 1, most of the known target genes of these two transcription factors are also upregulated during sake fermentation by X2180, whereas they exhibit lower expression levels in K701, predominantly in the earlier stages of fermentation (days 2 to 5). This period is comparable to that in the small-scale sake brewing experiments when the peak fermentation rate was observed. Together, these findings indicate that Adr1p and Cat8p are temporarily inactivated in sake yeast both in aerobic YPD culture and under anaerobic fermentative conditions.

Deletion of ADR1 and CAT8 increased the fermentation rate of laboratory yeast

To determine the importance of Adr1p and Cat8p inactivation in alcoholic fermentation, we performed fermentation tests in YPD medium containing 20% glucose using Δ adr1, Δ cat8, and Δ adr1 Δ cat8 disruptants against a laboratory yeast background (Fig. 2). Although the wild-type X2180-1A haploid strain and the Δ adr1 and Δ cat8 single disruptants did not show clear differences in fermentation profiles, the fermentation rate of the Δ adr1 Δ cat8 double mutant was markedly increased (total fermentation time, 5.38 d for the wild type; 5.27 d for Δ adr1, 5.26 d for Δ cat8; and 4.73 d for Δadr1 Δcat8, a 12.0% reduction compared to that of the wild-type strain). Although the peak fermentation rates were similar among the wild-type and mutant strains (201.7 mL/6 h for wild type; 187.1 mL/6 h for Δadr1; 220.8 mL/6 h for Δcat8; 212.1 mL/6 h for Δadr1 Δcat8), the fermentation rate of Δadr1 Δcat8 exceeded that of the wild-type strain by more than 20% from day 2.0 to day 3.5 after the onset of fermentation (Fig. 2). These results indicate that Adr1p and Cat8p cooperatively inhibit alcoholic fermentation, particularly after the peak fermentation rate is reached. In addition, deletion of the SNF1 gene, which encodes the upstream regulator for Adr1p and Cat8p, did not increase the fermentation rate of laboratory strain BY4743 under similar conditions (data not shown). Thus it appears that the activation of Adr1p and Cat8p by unknown factors might be more important than their activation by Snf1p in the negative control of alcoholic fermentation in S. cerevisiae.

Next, we confirmed that deletion of the ADR1 and CAT8 genes also improved sake fermentation by X2180-1A (Fig. 3). Compared to the wild-type strain, the double disruptant strain Δ adr1 Δ cat8 exhibited a lower fermentation rate in the early stage (1 to 2 d), a similar peak fermentation rate after 3 to 4 d, and statistically, a significantly higher fermentation rate after the peak fermentation rate was reached (5 to 17 d). This pattern is consistent with the fermentation profiles of Δadr1 Δcat8 observed in YPD medium (Fig. 2). Analysis of the finished sake after 20 d of fermentation revealed that the sake produced by the double disruptant had a higher ethanol concentration and a lower specific gravity than that produced by the wild-type strain, indicating that the deletion of ADR1 and CAT8 had a positive effect on alcoholic fermentation (Table 3). Together, our findings support the hypothesis that inactivation of the key transcription factors in glucose derepression contributes to an acceleration of alcoholic fermentation in sake yeast.

### Table 2. qRT-PCR Analysis of ADH1 and ADH2 mRNA Levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strain</th>
<th>Expression level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH1</td>
<td>WT</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Δ adr1</td>
<td>81.2</td>
</tr>
<tr>
<td></td>
<td>Δ rim15</td>
<td>86.1</td>
</tr>
<tr>
<td>ADH2</td>
<td>WT</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Δ adr1</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>Δ rim15</td>
<td>484.3</td>
</tr>
</tbody>
</table>

*Relative expression levels of the ADH1 and ADH2 genes in cells grown in YPD medium for 7 d are shown as percentages compared to the levels in the wild type (WT), using ACT1 as reference gene. The data represent averaged values from two independent experiments that showed similar results.*
Disrupting \( ADR1 \) and \( CAT8 \) had a statistically significant but only slight effect in improving fermentation performance. As for the ethanol concentration after 20 days of sake fermentation, \( \Delta adr1 \Delta cat8 \) rose by only 5.0% against the X2180-1A background (Table 3), while \( \Delta rim15 \) increased by 52.2% against the BY4743 background.\(^3\) Thus inactivation of the Rim15p-Msn2/4p pathway accounts for most of the high fermentation ability of modern sake yeast strains. \( \Delta adr1 \Delta cat8 \) increased the fermentation rate specifically in the later stages of fermentation (Figs. 2 and 3), whereas \( \Delta rim15 \) mainly enhances the peak fermentation rates.\(^3\) Hence we hypothesize that multiple causal mutations in various signalling pathways independently or synergistically contribute to the high fermentation rate of sake yeast throughout the fermentation period. Our previous identification of at least 17 quantitative trait loci (QTLs) affecting fermentation in K7\(^4\) confirms this hypothesis. Novel sake yeast-specific mutations and genes associated with the control of alcoholic fermentation and their interaction with \( \Delta adr1 \Delta cat8 \) and/or \( \Delta rim15 \) should be further investigated.

How does dysfunction of Adr1p and Cat8p result in increased ethanol production rates? The most plausible explanation is that induction of the genes related to ethanol utilization, including \( ADH2, ALD4, ACS1, \) and \( CIT3 \), allows yeast cells to consume accumulated ethanol in the sake mash as an alternative carbon and

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**Fig. 2.** Deletion of Both the \( ADR1 \) and the \( CAT8 \) Gene Improved the Fermentation Rate. Generation of carbon dioxide gas was measured during the fermentation tests in a 20% glucose-containing YPD medium using the wild type (gray) and the \( \Delta adr1 \) and/or \( \Delta cat8 \) mutants (black) against the X2180-1A background. Total amounts of carbon dioxide evolved (top) and amounts of carbon dioxide emission every 6 h (bottom) are shown. Averaged data from three independent experiments are shown. WT, wild type. \(^a\)Significantly lower than the wild type (\( t \) test, \( p < 0.05 \)). \(^b\)Significantly higher than the wild type (\( t \) test, \( p < 0.05 \)).

**Fig. 3.** Deletion of Both the \( ADR1 \) and the \( CAT8 \) Gene Improved the Fermentation Rate in Sake Mash. Generation of carbon dioxide gas was measured during small-scale sake brewing tests using the wild type (gray) and the \( \Delta adr1 \Delta cat8 \) double mutant (black) against the X2180-1A background. Total amounts of carbon dioxide evolved (left) and the amounts of carbon dioxide emission every 24 h (bottom) are shown. Averaged data from three independent experiments are shown. WT, wild type. \(^a\)Significantly higher than the wild type (\( t \) test, \( p < 0.05 \)). \(^b\)Significantly higher than the wild type (\( t \) test, \( p < 0.05 \)).

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energy source, even under fermentative conditions. To prove this conclusively, it should be investigated from a metabolic viewpoint whether glucose derepression-like ethanol consumption is induced in fermenting sake mash that is highly anaerobic and contains abundant glucose.

In addition, we speculate that dynamic changes in gene expression caused by Adr1p and Cat8p22,23) negatively affect intracellular carbon metabolism toward ethanol production, as Rim15p does. Inactivation of the Rim15p-Msn2/4p pathway probably contributes to an improvement of fermentation rates through repression of genes related to stress-responsive carbohydrate metabolism (e.g., trehalose and glycogen synthesis), which diversifies the cellular glucose flux (reference 3 and D. Watanabe et al., unpublished results). Since the target genes of Adr1p and Cat8p include peroxisomal biogenesis, amino acid transport, fatty acid biogenesis, glycogen biogenesis, and cell wall organization-related genes, as well as ones involved in non-fermentative carbon metabolism,22,23) the global effects of Adr1p and Cat8p on yeast cellular metabolism should be revealed by metabolomic analysis in the future.

Another striking feature of the sake produced by the Δadr1 Δcat8 double disruptant was a higher acidity level, which was apparently caused by increased levels of acetic and lactic acid (Table 3). Since activation of Adr1p and Cat8p also causes induction of the genes involved in the utilization of acetic and lactic acid, including ADY2, ACH1, JEN1, and DLD1, deletion of the ADR1 and CAT8 genes might lead to decreased consumption of these organic acids by yeast cells during sake brewing.

**Table 3. Effects of Deletion of the ADR1 and CAT8 Genes on Selected Sake Brewing Characteristics of Laboratory Yeast Strain X2180-1A**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>Δadr1 Δcat8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (vol %)</td>
<td>15.30 ± 0.06</td>
<td>16.07 ± 0.06*</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.0287 ± 0.0003</td>
<td>1.0257 ± 0.0003*</td>
</tr>
<tr>
<td>pH 7.2</td>
<td>4.23 ± 0.06</td>
<td>4.54 ± 0.03*</td>
</tr>
<tr>
<td>Amino acid level</td>
<td>1.89 ± 0.05</td>
<td>1.96 ± 0.05</td>
</tr>
<tr>
<td>Citric acid (mg/L)</td>
<td>76.8 ± 0.8</td>
<td>79.2 ± 1.4</td>
</tr>
<tr>
<td>Malic acid (mg/L)</td>
<td>116.7 ± 1.7</td>
<td>107.6 ± 2.4*</td>
</tr>
<tr>
<td>Succinic acid (mg/L)</td>
<td>374.5 ± 7.4</td>
<td>369.6 ± 5.9</td>
</tr>
<tr>
<td>Pyruvic acid (mg/L)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acetic acid (mg/L)</td>
<td>1089.3 ± 9.7</td>
<td>1150.5 ± 14.0*</td>
</tr>
<tr>
<td>Lactic acid (mg/L)</td>
<td>343.5 ± 3.1</td>
<td>456.9 ± 3.8*</td>
</tr>
<tr>
<td>Isoamyl acetate (ppm)</td>
<td>0.63 ± 0.03</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>Ethyl caproate (ppm)</td>
<td>0.73 ± 0.03</td>
<td>0.78 ± 0.03</td>
</tr>
</tbody>
</table>

*All of the selected parameters were analyzed as previously reported.53)
*Significantly higher than the wild type (WT) (t test, p < 0.01).
*Significantly lower than WT (t test, p < 0.01).

We examined the nucleotide sequences of the ADR1 and CAT8 genes for mutations. Using the whole-genome sequence of diploid sake yeast strain K7,43) we discovered a loss-of-function mutation in both copies of the ADR1 gene. Since the single A-to-T point mutation identified at nucleotide 658 changes a lysine codon (AAG) into a stop codon (TAG) (K220*, Fig. 4B), three transcription activation domains (TADs), respectively. TAD I, overlapping with PAR and ZFs, is represented by a blue line. Two phosphorylation sites are also shown. White and red dots indicate the positions of non-synonymous mutations. B. Nucleotide and amino acid sequences surrounding the adr1Δ mutation of K7 (red letters). C. Nucleotide and amino acid sequences surrounding the adr1Δ mutation of K701 (red letters). Conserved cysteine and histidine residues in the Cys2-His2-type zinc-finger motif are indicated by blue letters.

**Fig. 4. Loss-of-Function Mutation in the ADR1 Gene of Sake Yeast Strains.**

A. Comparison of products of the ADR1 gene among laboratory strain S288c and existing sake strains K7, K701, and K11. Green, orange, and blue boxes represent a proximal accessory region (PAR), zinc-finger motifs (ZFs), and transcriptional activation domains (TADs), respectively. TAD I, overlapping with PAR and ZFs, is represented by a blue line. Two phosphorylation sites are also shown. White and red dots indicate the positions of non-synonymous mutations. B. Nucleotide and amino acid sequences surrounding the adr1Δ mutation of K7 (red letters). C. Nucleotide and amino acid sequences surrounding the adr1Δ mutation of K701 (red letters). Conserved cysteine and histidine residues in the Cys2-His2-type zinc-finger motif are indicated by blue letters.
and C109A,51 exhibit severely decreased DNA-binding activity, leading to an undetectable level of Adh2p activity. It is thus likely that the adr1pT225A mutation also causes dysfunction of Adr1p in K701. This mutation might at least partly explain the defective Adr1p-mediated gene expression observed in K701 (Table 1, Fig. 1, and Supplemental Fig. 1). No other obvious loss-of-function mutations were found in the ADR1 gene of K11 or in the CAT8 genes of K7, K701, and K11.

Our findings raise a question: how did K7 and K701 acquire the different loss-of-function mutations in the ADR1 gene? We assume that the ancestral K7 strain is genetically distinguishable from the existing K7 strain (Supplemental Fig. 2). In the ancestral K7 strain, both Adr1p and Cat8p might have been inactivated indirectly by mutations in their unidentified upstream regulator to inhibit glucose derepression that interferes with alcohol fermentation. After isolation of K701 and K11 from the ancestral K7 strain in the 1970s,27,28 each strain might have independently accumulated loss-of-function mutations during repeated subculture over the decades. Although the common mutations that have inactivated Adr1p and Cat8p among the existent K7, K701, and K11 strains should be investigated, the presence of different types of loss-of-function mutations between K7 and K701 strongly indicates that the ADR1 gene is no longer required for sake yeast strains to perform alcoholic fermentation effectively. To verify this, inactivation of Adr1p and Cat8p should be checked in K11, in which no apparent loss-of-function mutations have been found in the ADR1 or the CAT8 gene.

In conclusion, our results suggest that downregulation of Adr1p/Cat8p-dependent genes plays a role in improving the alcoholic fermentation rate of sake yeast in the later stages of fermentation. We have found that defects in the stress responses mediated by Rim15p,33 Msn2/4p,4 and Hsf1p52 contribute to enhancement of the initial fermentation rate in modern sake yeast strains. These findings, together with our present results, indicate that sake yeast has multiple cellular functions that have been impaired during years of selection and domestication, and that are unnecessary for or negatively affect efficient alcoholic fermentation. The genetic approach used here to analyze sake yeast strains helps elucidate dispensable regulatory mechanisms for alcoholic fermentation by S. cerevisiae, and aids in the development of industrial yeast strains with desirable fermentation properties.

Acknowledgments

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References