**Triggering Respirofermentative Metabolism in the Crabtree-Negative Yeast *Pichia guilliermondii* by Disrupting the *CAT8* Gene**

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*Pichia guilliermondii* is a Crabtree-negative yeast that does not normally exhibit respirofermentative metabolism under aerobic conditions, and methods to trigger this metabolism may have applications for physiological study and industrial applications. In the present study, *CAT8*, which encodes a putative global transcriptional activator, was disrupted in *P. guilliermondii*. This yeast’s ethanol titer increased by >20-fold compared to the wild type (WT) during aerobic fermentation using glucose. A comparative transcriptional analysis indicated that the expression of genes in the tricarboxylic acid cycle and respiratory chain was repressed in the CAT8-disrupted (ΔCAT8) strain, while the fermentative pathway genes were significantly upregulated. The respiratory activities in the ΔCAT8 strain, indicated by the specific oxygen uptake rate and respiratory state value, decreased to one-half and one-third of the WT values, respectively. In addition, the expression of *HAP4*, a transcriptional respiratory activator, was significantly repressed in the ΔCAT8 strain. Through disruption of *HAP4*, the ethanol production of *P. guilliermondii* was also increased, but the yield and titer were lower than that in the ΔCAT8 strain. Further transcriptional comparison between ΔCAT8 and ΔHAP4 strains suggested a more comprehensive reprogramming function of *Cat8* in the central metabolic pathways. These results indicated the important role of *CAT8* in regulating the glucose metabolism of *P. guilliermondii* and that the regulation was partially mediated by repressing *HAP4*. The strategy proposed here might be applicable to improve the aerobic fermentation capacity of other Crabtree-negative yeasts.

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this study. The physiology of the CAT8 mutant and the wild-type strain were compared. The effect of CAT8 disruption and its possible role in regulating respiratory/fermentative metabolism were analyzed and discussed.

**MATERIALS AND METHODS**

**Strains and media.** The parental *P. guilliermondii*, which is an inhibitor-tolerant strain isolated and maintained in our laboratory (3), was maintained as described previously (4). YNB medium was used for the molecular biology experiments (17), and 0.05 g of uracil liter~1~ and/or 1 g of 5-fluoroorotic acid (5-FOA) liter~1~ was supplemented for selection purposes. The uracil auxotrophic strain was obtained via spontaneous mutation and selected on YNB plates supplemented with uracil and 5-FOA (Sigma-Aldrich Co., St. Louis, MO) as reported (17). Nearly 200 colonies, which resisted 5-FOA and could not grow without uracil addition, were obtained. After these colonies were passed five times on uracil-supplemented YNB plates (without 5-FOA), only one mutant maintained the uracil auxotrophic physiology. This stable mutant was further cultivated in liquid YNB medium supplemented with uracil, and its ura3 gene was then cloned and verified by sequencing. A point mutation at bp 314 was inserted into the ura3 gene, even though the resultant mRNA cannot express the correct CAT8 protein due to a lack of stop codon, even though the CAT8 gene could start the expression.

Transformants were selected on uracil-deficient (Ura−) YNB plates, and the positive colonies were selected. The selected colonies were plated on new Ura− YNB plates, and the correct insertion was verified by colony PCR using the primers P1 and P2 (Fig. 1B and C). The insertion correctness was further verified by sequencing the PCR product.

**Analysis of cell growth and fermentation metabolites.** Samples were taken at the designated time to analyze the cell concentration, 0.2 ml of fermentation broth was taken and diluted 25-fold using deionized water. The optical density at 620 nm (OD620) for each sample was measured (1700Pharmaspec UV spectrophotometer; Shimadzu, Japan) and correlated with the dry cell weight (DCW) (4).

To analyze the metabolites, 0.5 ml of fermentation broth was withdrawn and immediately centrifuged (5804R centrifuge; Eppendorf, Hamburg, Germany) at 10,000 × g for 5 min at 4°C. The supernatants were filtered through 0.22-μm-pore-size filters and stored at ~2°C until high-pressure liquid chromatography (HPLC) analysis. The concentrations of glucose, glycerol, pyruvate, ethanol, and acetic acid were detected in an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) with a refractive index detector and a UV (210-nm) detector using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 65°C. The mobile phase and its flow rate were 5 mM H2SO4 and 0.6 ml min~−1~, respectively.

<table>
<thead>
<tr>
<th>Strains, plasmid, or primer</th>
<th>Description or sequence (5′-3′)a</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
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<td>Parental strain</td>
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<tr>
<td>WT</td>
<td>URA− <em>P. guilliermondii</em></td>
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</tr>
<tr>
<td>ΔCAT8 mutant</td>
<td>CAT8-disrupted WT</td>
<td>This study</td>
</tr>
<tr>
<td>ΔHAP4 mutant</td>
<td>HAP4-disrupted WT</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pAGU34</td>
<td>pUC19 with URA3 from <em>S. cerevisae</em></td>
<td>17</td>
</tr>
<tr>
<td>pAGUARS</td>
<td>pAGU34 with ARS deleted</td>
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<tr>
<td>pCATIN</td>
<td>pAGUARS ligated with <em>P. guilliermondii</em> CAT8 fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pHAPIN</td>
<td>pAGUARS ligated with <em>P. guilliermondii</em> HAP4 fragment</td>
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<tr>
<td>HP2</td>
<td>TAAATTTCTTGTGTGTAAGAAACATCC</td>
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*a Underlined sequences indicate the recognition sites of DNA restriction enzymes described in Materials and Methods.*
At the designated fermentation time points, a 20-ml portion of culture from three independent flasks of each condition was used for mRNA extraction. The fermentation broth was sampled and immediately centrifuged (3,000 × g, 5 min) at 4°C. The cell pellets were immediately frozen in liquid nitrogen and stored at −80°C until use. For all extracted mRNA samples, the OD_{260}/OD_{280} ranged from 1.8 to 2.2.

**Measurement of oxygen consumption.** The dissolved oxygen (DO) was measured using a dissolved oxygen probe (DO-18; East China University of Science and Technology, Shanghai, China) inserted into a 100-ml flask with a port at the bottom containing 40 ml of MM medium. The flask was placed on a rotary shaker operating at 150 rpm and 30°C. When analyzing the oxygen uptake rate, the cells were collected from 20 ml of medium at designated time points and suspended in 40 ml of fresh MM medium with 20 g of glucose liter⁻¹. Pure oxygen was gassed into the liquid through a silicone tube. When the oxygen concentration was close to air saturation, the flask port was sealed with a rubber stopper. The oxygen uptake rate was estimated from the DO slope as a function of time.

**Calculation.** The following equation was used to calculate the ethanol yield from sugar (glucose). The calculations were based on the beginning to the peak (h 32) of ethanol production for the mutant. The ethanol productivity and glucose consumption rate were calculated by using the following equation. The calculations were based on the beginning of the calculation period, respectively, and RSV was then calculated from these values using the following equation: 

\[
RSV = \frac{C_{t2} - C_{t1}}{t_2 - t_1}
\]

where \(C_{t1}\) and \(C_{t2}\) represent the glucose concentrations at the beginning and end of the calculation period, respectively, and \(t_1\) and \(t_2\) represent the ethanol concentrations at the beginning and end of the calculation period, respectively.

**Statistical analysis.** The data consisted of the average of the measurements of three independent experiments. Error bars represent the standard deviation. A P value of <0.05 was considered statistically significant.
standard deviations from the means of triplicates. Statistical significance was analyzed using Student t test, where P < 0.05 indicates significance.

RESULTS

Disruption of CAT8 gene in P. guilliermondii. The genome of P. guilliermondii, a haploid yeast, was sequenced and annotated by Butler et al. (20), in which the CAT8 gene is identified. In the present study, a CAT8-disrupted mutant was obtained via homologous recombination. Six correct homologous recombinants were identified from nearly 900 transformants, and the remaining transformants were identified as illegitimate recombinants. To test the stability, a selected CAT8-disrupted mutant was cultivated in liquid uracil-supplemented YNB medium for 5 passages. Ura\textsuperscript{+} prototroph and correct PCR products were confirmed in each passage (Fig. 1D). To test the possible reversion mutagenesis, a droplet of liquid culture from the fifth passage was plated on a YNB plate to obtain more than 500 colonies. Next, 500 colonies were selected and replated on five YNB plates with 5-FOA and uracil. After 1 week, no colony was observed in any of the plates, which indicated a low level of reversion mutagenesis rate for the CAT8 mutant. In summary, CAT8 was successfully disrupted, and a stable \( \Delta \text{CAT8} \) mutant was obtained.

The transformation cassette used in the present study did not contain a yeast autonomously replicating sequence; thus, the functional expression of URA3 gene can only occur after genomic integration (21). The homologous recombination rate of P. guilliermondii could be as low as 1/1,000 (17). In the present study, the insertion mutagenesis strategy with ends-in vector was used, which was reported to yield higher homologous recombinant rate in yeast (22), and resulted in a homologous integration rate of 1/150.

Triggered respirofermentative metabolism in the \( \Delta \text{CAT8} \) mutant versus the WT. The fermentation performance of the wild type (WT) and the CAT8-disrupted mutant (\( \Delta \text{CAT8} \)) of P. guilliermondii were compared during aerobic cultivation in minimum medium with 30 g of glucose liter\textsuperscript{-1} as a carbon source and ammonium as a nitrogen source (Fig. 2). The WT showed typical respirative characteristics; glucose was completely consumed in 46 h with little ethanol formation. The ethanol production was significantly enhanced in the \( \Delta \text{CAT8} \) mutant for the same aerobic condition (Fig. 2C). The \( \Delta \text{CAT8} \) mutant reached an ethanol titer of 4.32 ± 0.13 g liter\textsuperscript{-1}, which was much higher than that of the WT. Moreover, the ethanol yield, productivity, and glucose consumption rate were all significantly increased for the mutant. Acetate and pyruvate also accumulated at higher levels in the \( \Delta \text{CAT8} \) mutant (Fig. 2B), suggesting an increased pyruvate decarboxylase (PDC) flux and a possible pyruvate overflow metabolism (23). The DCW in the \( \Delta \text{CAT8} \) mutant was only 70% of that in the WT when the glucose was depleted in the medium. Moreover, the titer of glycerol, a major by-product during ethanol production, was only 30% of that of WT (Fig. 2C).

The above findings suggest that the disruption of CAT8 efficiently triggered the respirofermentative metabolism of P. guilliermondii under aerobic conditions. To further understand the influence of CAT8 disruption, the transcriptional levels of the metabolic pathway genes are analyzed below.

Transcriptional analysis of key genes in glucose metabolism of the WT and the \( \Delta \text{CAT8} \) mutant. The glucose consumption and ethanol production rates reached and maintained almost constant levels from the 24th to the 32nd hour for both WT and \( \Delta \text{CAT8} \) fermentation (Fig. 2). To elucidate the transcriptional differences in the central carbon metabolism between the WT and \( \Delta \text{CAT8} \) strains, the expression levels of marker genes were analyzed by qRT-PCR at 24, 28, and 32 h.

As shown in Table 2, the expression levels of genes in the central metabolic pathways changed significantly in the WT during aerobic glucose fermentation. From 24 to 32 h, the expression of CAT8 gradually increased by ≥2-fold. The expression of putative HAP4 (PGUG_03337), a positive respiratory regulator in yeast
(14, 24, 25), also increased 2.8-fold from 24 to 32 h. Coinciding with the activation of these two regulators, the tricarboxylic acid (TCA) cycle and respiratory gene expression levels also increased by 3- to 6-fold, respectively. Possibly due to the activation of TCA cycle, the ethanol titer (at 0.2 g liter\(^{-1}\)) was not further increased after 24 h in the WT. Meanwhile, the biomass productivity of the WT increased to a higher level and surpassed that of the \(\Delta CAT8\) mutant (Fig. 2A). In the \(\Delta CAT8\) mutant, most of the genes (except \(pdc\)) in the metabolic pathways were expressed at stable levels, and significant differences at the transcriptional levels of genes were not observed between 24 and 32 h. At 32 h, nearly all of the glucose was completely consumed and the ethanol titer was maximized in the \(\Delta CAT8\) mutant. Possibly due to the consumption of ethanol, the gene expression levels at 32 h showed a pattern different than those at 24 and 28 h in the \(\Delta CAT8\) mutant, where respiratory genes were upregulated for ethanol metabolism.

When we compared between the WT at 32 h and the \(\Delta CAT8\) mutant at 24 h, which were, respectively, in their middle stages of glucose fermentation, we found the expression of pyruvate dehydrogenase (\(pdc\)), alcohol dehydrogenase (\(adh\)), and acetaldehyde dehydrogenase (\(aldh\)) to be 3.9-, 3.2-, and 5.3-fold higher, respectively, in the \(\Delta CAT8\) mutant. In the TCA cycle, \(sdh\), \(fum\), and \(mdh\), which encode enzymes that catalyze the reactions from succinate to oxaloacetate, were 2.7-, 3.2-, and 2.0-fold lower, respectively, in the \(\Delta CAT8\) mutant relative to the WT. The TCA cycle is linked to the mitochondrial respiratory chain by succinate dehydrogenase (SDH) and cytochrome \(c\) reductase via ubiquinone-mediated electron transfer. The expression of a gene that encodes a subunit of cytochrome \(c\) reductase, \(qcr\), was also 1.8-fold lower in the \(\Delta CAT8\) mutant.

The results of transcriptional analysis indicate that the \(CAT8\) disruption led to a repressed respiratory system. In contrast to the WT, the expression level of \(HAP4\) was not activated in the \(\Delta CAT8\) mutant during glucose fermentation and was 2.3-fold lower than in the WT. The activation of \(HAP4\) transcription by \(CAT8\)p was proven by using an electrophoretic mobility shift assay in \(S. cerevisiae\) (26). The \(Cat8\) protein of \(P. guilliermondii\) contains a similar GAL4 zinc cluster DNA-binding domain with an identity of 73% compared to \(S. cerevisiae\), possibly indicating similar binding characteristics. In addition, a sequence (CCCAAACACCG) similar to the \(CAT8\)-specific binding site (CCNNNNNNCCG) was found in the promoter of the \(HAP4\) (27). These facts indicate that \(HAP4\) might be regulated by \(CAT8\) in \(P. guilliermondii\); thus, \(CAT8\) disruption repressed \(HAP4\). Whether the respiratory activity decreased in the \(\Delta CAT8\) mutant and whether \(HAP4\) can mediate the repression of the respiratory chain were tested in the following experiments.

### Fermentation and respiratory activity of WT, \(CAT8\), and \(HAP4\) disruption during aerobic glucose fermentation

To test the influence of \(HAP4\) on metabolism, a \(HAP4\) disruption mutant (\(\Delta HAP4\)) was constructed using the same method used to construct the \(\Delta CAT8\) mutant. The primers used to clone the homologous region and verify the correct recombination are listed in Table 1. A correct \(HAP4\) disruption mutant was obtained and verified by sequencing according to the same protocol used for the \(CAT8\) disruption.

The glucose fermentation products were analyzed, and similar pattern were observed in the \(\Delta CAT8\) and \(\Delta HAP4\) mutants (Fig. 3). The cells and glycerol produced from glucose decreased by 20 and 60%, respectively, in the \(\Delta HAP4\) strain compared to the WT. Similar to the \(\Delta CAT8\) mutant, the ethanol productivity and glucose consumption rate significantly increased in the \(\Delta HAP4\) mutant. \(HAP4\) disruption also resulted in increased ethanol, pyruvate, and acetate accumulation.

The effects of \(CAT8\) or \(HAP4\) disruption on the respiratory properties were characterized by analyzing the specific oxygen uptake rates in the middle phase of glucose fermentation for the WT, \(\Delta HAP4\), and \(\Delta CAT8\) strains at 32, 24, and 24 h of fermentation.
respectively. As shown in Table 3, the basal and maximum oxygen uptake rate ($I_{O2}$ and $I_{max}$ respectively) of the $\Delta$CAT8 mutant decreased by up to 50% compared to those of the WT. The addition of TET, a lipophilic $F_0F_1$-ATPase inhibitor, did not reduce the specific oxygen uptake rate in the $\Delta$CAT8 mutant, which indicated that the activity of oxidative respiration was very low in this mutant. The respiration state value (RSV), which represents the oxidative phosphorylation efficiency, was then calculated. The RSV of the $\Delta$CAT8 mutant was only one-third that in the WT. A reduction in respiratory activity was also observed in the $\Delta$HAP4 mutant (Table 3). The $I_{O2}$ and $I_{max}$ of the $\Delta$HAP4 mutant showed low levels similar to those observed for the $\Delta$CAT8 mutant, and TET did not affect the oxygen uptake rates. The RSV of the $\Delta$HAP4 mutant was even lower than that of the $\Delta$CAT8 mutant.

The results described above indicate that HAP4 is an important respiratory regulator in P. guilliermondii. Considering the 2.8-fold-lower expression of HAP4 in the $\Delta$CAT8 mutant, it was possible that the effects of CAT8 disruption on the respiratory activity could be partly attributed to its repression of HAP4.

It is noteworthy that although the respiratory efficiency was higher in the $\Delta$CAT8 mutant (as indicated by the higher RSV) than in the $\Delta$HAP4 mutant, the ethanol yield was significantly higher and cell yield was lower in the $\Delta$CAT8 mutant (Table 3). It is quite possible that in the $\Delta$CAT8 mutant, in addition to the repression of respiration, the influences of other pathways also contributed to efficient glucose-ethanol transformation. The transcriptional differences in the central metabolic pathways were then compared between the $\Delta$HAP4 and $\Delta$CAT8 strains to determine the differences between these two mutants.

Comparison of gene expression in central metabolism pathways in the $\Delta$HAP4 and $\Delta$CAT8 mutants during aerobic glucose fermentation. The expressions of marker genes of the central metabolism pathways were analyzed by qRT-PCR and compared between the $\Delta$HAP4 and $\Delta$CAT8 mutants in the middle phase of glucose consumption (i.e., at 24 h). The values were normalized by the expression levels of WT (at 32 h). Values higher than 1 indicated higher expression levels compared to the WT and vice versa. As shown in Fig. 4, the expression levels of the glycolysis pathway genes were significantly lower in the $\Delta$CAT8 mutant than in the $\Delta$HAP4 mutant, and the expression levels of these genes in both mutants were lower than that of the WT. The expression levels of QCR, sco, sdh, and $\mu$fum, which are closely related to the respiratory pathway, were similar in the $\Delta$CAT8 and $\Delta$HAP4 mutants. The expression levels of other TCA genes, such as cit, aco, and idh, were lower in the $\Delta$CAT8 mutant than in the $\Delta$HAP4 mutant. In particular, the genes in the glyoxylate and anaplerotic pathways, including mls, mdh, pyc, and pepck, were repressed in the $\Delta$CAT8 mutant but not in the $\Delta$HAP4 mutant.

TABLE 3 Yields and respiratory parameters of $\Delta$CAT8, $\Delta$HAP4, and WT strains during aerobic glucose fermentation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell yield (g g$^{-1}$)</th>
<th>Ethanol yield (g g$^{-1}$)</th>
<th>$I_{O2}$</th>
<th>$I_{max}$</th>
<th>RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.54 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>1.11 ± 0.10</td>
<td>0.87 ± 0.18</td>
<td>1.45 ± 0.06</td>
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<tr>
<td>$\Delta$HAP4 mutant</td>
<td>0.35 ± 0.05*</td>
<td>0.21 ± 0.02*</td>
<td>0.57 ± 0.08*</td>
<td>0.56 ± 0.08*</td>
<td>0.75 ± 0.01*</td>
</tr>
<tr>
<td>$\Delta$CAT8 mutant</td>
<td>0.23 ± 0.02*†</td>
<td>0.26 ± 0.01*†</td>
<td>0.50 ± 0.06*</td>
<td>0.47 ± 0.07*</td>
<td>0.67 ± 0.04*</td>
</tr>
</tbody>
</table>

$^a$In the WT strain, the yields were calculated from 16 to 50 h, and respiratory activity was analyzed at 32 h during fermentation. In the $\Delta$HAP4 and $\Delta$CAT8 mutant strains, the yields were calculated from 16 to 26 h, and respiratory activity was analyzed at 24 h during fermentation.

$^b$The averages of three independent experiments are shown. *, statistically significant difference (Student t test [paired, two tailed], P < 0.05) compared to the value for the WT; †, statistically significant difference (Student t test [paired, two tailed], P < 0.05) between the values for the $\Delta$HAP4 and $\Delta$CAT8 mutants.

FIG 3 Aerobic glucose fermentation of $\Delta$CAT8 (closed symbols), $\Delta$HAP4 (open symbols), and WT (dash lines with closed gray symbols) strains at 30°C and 200 rpm in 100-ml Erlenmeyer flasks with 20 ml of MM medium. (A) Time courses of medium residual glucose (squares) and DCW (circles). (B) Time courses of pyruvate (triangles) and acetate (squares) formation. (C) Time courses of ethanol (squares) and glycerol (circles) formation. The values are the averages of three independent experiments, and the error bars represent the standard derivation. Statistically significant differences (Student t test [paired, two tailed], P < 0.05) were determined by using the Student t test and are indicated with an asterisk.
showed similar patterns in the respiratory- and ethanol fermentation-related genes generally. Glucose fermentation of the more comprehensive regulatory responses related to TCA cycle, which favored the fermentative metabolism in both mutants. However, due to the role of CAT8p as a global regulator and the repression of glyoxylate and anaplerotic pathways were observed in the ΔCAT8 mutant, which might provide better fermentative characteristics than for the ΔHAP4 mutant.

**DISCUSSION**

Engineering the respirative metabolism in Crabtree-negative yeasts will broaden the current knowledge of the respiratory machinery and assist the construction of these yeasts as potential industrial organisms to produce ethanol and other valuable fermentative products (9). Little is known of the regulation of aerobic glucose metabolism in *Pichia* (28), possibly because the genome of *Pichia* strain was only sequenced and annotated recently (20). The results presented here demonstrated that the engineering of a putative global regulator was effective in shifting the central carbon flux in the *P. guilliermondii*, which is an important biochemical producer. Furthermore, our findings indicated that the respirofermentative metabolism during aerobic glucose fermentation could be triggered by engineering the glucose repression signaling cascade.

By disrupting CAT8, the glucose metabolism was systematically shifted toward a fermentative metabolism at both the metabolic and the transcriptional levels. The expression levels of genes in the TCA cycle and respiratory chain were lower in the mutant than in the WT. Moreover, the oxygen uptake rate and respiratory efficiency were significantly reduced. In yeast, the mitochondrial content was strictly adjusted and linearly correlated with the oxygen uptake rate (29); thus, the disruption might also affect the physiology of mitochondria. These results suggested that the disruption of CAT8 affected the activity of the TCA cycle and respiratory chain. One possible explanation was that the HAP4 gene, which encodes a transcriptional activator for the TCA cycle and respiratory chain (14, 24, 25), was repressed in the ΔCAT8 mutant.

When HAP4 was disrupted, the expression levels of respiratory-related genes were severely reduced compared to the WT during aerobic glucose fermentation. Meanwhile, the expression of ethanol production-related genes, such as *pdc* and *adh*, increased in the ΔHAP4 mutant. As indicated by the RSV, the respiratory efficiency was reduced to even lower levels in the ΔHAP4 mutant compared to the ΔCAT8 mutant (16). As a result, the fermentative metabolism was greatly enhanced in the ΔHAP4 mutant, which proved the importance of HAP4 in regulating the glucose metabolism of *P. guilliermondii*. Based on these results, it is quite possible that the repression of HAP4 could be one of the reasons for the enhanced fermentative metabolism in the ΔCAT8 mutant.

Interestingly, the ethanol yield and productivity of the ΔCAT8 mutant were higher than those of the ΔHAP4 mutant. CAT8 disruption was noted to lead to a more comprehensive reprogramming of the central metabolism pathways than HAP4 disruption. A comparison between the expression profiles of the ΔCAT8 and ΔHAP4 mutants showed that the gene expression levels of the glyoxylate and anaplerotic pathways were lower in the ΔCAT8 mutant. These two pathways are important for replenishing the TCA cycle flux and providing building blocks for cell formation. Their repression may further promote the fermentative metabolism in the ΔCAT8 mutant. This fact hinted at the possibility of further improving the ethanol fermentation of *P. guilliermondii*. In a preliminary test in our lab, the inhibition of pyruvate carboxylase increased the ethanol yield and productivity of the ΔCAT8 strain (data not shown). Reducing the expression of the related
genes by substituting their promoters with weaker ones would be important to clarify the significance of the glyoxylate and/or anaerobic pathways and further improve the ethanol production in future work. Because the TCA cycle is one of the most important pathways for cell metabolism, the direct deletion of individual enzymes in this pathway could be detrimental to yeast (30). Fortunately, the TCA cycle genes in the ΔCAT8 mutant were repressed ~2-fold compared to the WT, which was not detrimental but possibly sufficient to improve the fermentative metabolism.

Because the repression of the TCA cycle and respiratory chain may also result in an NAD(P)H imbalance, a new redox related reaction should be activated to maintain a balanced metabolism. The activated fermentative pathway redirected the flux from the TCA cycle and also possibly fulfilled the function of balancing the redox. Compared to S. cerevisiae, respiratory yeasts always showed a higher TCA cycle flux and lower glycolytic flux (31). A study of S. cerevisiae showed that the repression of the TCA cycle resulted in a loss of ATP production. To meet the ATP demand, glycolysis was accelerated (32). In the present study, the glucose consumption rate was significantly enhanced in the ΔCAT8 mutant, which was a major reason for the increased ethanol productivity. Interestingly, the expression levels of glycolytic genes were much higher in the WT than the mutants despite the lower glucose consumption rate. A similar phenomenon was observed in a comparison of S. cerevisiae and its respiratory mutant; the expression levels of glycolytic genes were higher in the respiratory yeast (32). In the present study, the glucose consumption rate was significantly enhanced in the ΔCAT8 mutant, which was a major reason for the increased ethanol productivity. Interestingly, the expression levels of glycolytic genes were much higher in the WT than the mutants despite the lower glucose consumption rate.

In conclusion, we successfully triggered a respirofermentative metabolism in P. guilliermondii by disrupting CAT8. The results demonstrated the importance of the glucose sensing cascade in the Crabtree effect of P. guilliermondii and its improved performance as a bioenergy platform. Moreover, due to its simplicity, this strategy may be applied to other respirative yeasts to improve their fermentation efficiency and induce respirofermentative metabolism.

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