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Identification of metabolic engineering targets for improving glycerol assimilation ability of *Saccharomyces cerevisiae* based on adaptive laboratory evolution and transcriptome analysis

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Glycerol, a by-product of biodiesel production, has been utilized as a raw material for bioproduction. *Saccharomyces cerevisiae*, which has been used as a host microorganism for bioproduction, possesses the metabolic pathways for glycerol assimilation, but it cannot grow on glycerol as a carbon source. In this study, we identified metabolic engineering targets to improve the glycerol assimilation ability of *S. cerevisiae* based on adaptive laboratory evolution experiments using serial transfer of culture on glycerol and transcriptome analysis of the evolved cells using RNA-sequencing. The transcriptome data revealed that the upregulation of genes related to the tricarboxylic acid (TCA) cycle and oxidative phosphorylation contributed to the increased specific growth rate on glycerol during adaptive evolution. Furthermore, genes related to the pentose phosphate pathway were downregulated. Based on these observations, we identified metabolic engineering targets for improving glycerol assimilation. Overexpression of *HAP4*, which encodes one of the subunits of the Hap2p/3p/4p/5p transcription factor complex involved in the upregulation of the TCA cycle genes, or disruption of *RIM15*, which encodes a protein kinase related to the transcription regulator Gis1p, as well as overexpression of *STL1*, which encodes the glycerol/H^+ symporter, improved the growth of *S. cerevisiae* on glycerol as the main carbon source. Our results indicate that the engineering targets can be identified based on adaptive laboratory evolution and transcriptome analysis of the evolved cells, and that the glycerol assimilation ability of *S. cerevisiae* is indeed improved by engineering the identified targets.

[**Key words:** *Saccharomyces cerevisiae*; Glycerol assimilation; Adaptive laboratory evolution; Transcriptome; Metabolic engineering]

The budding yeast *Saccharomyces cerevisiae* has been used not only as a model organism in fundamental research, but also as a host microorganism for bioproduction. This is facilitated by the availability of various recombinant DNA and genome engineering tools for *S. cerevisiae* and the genome DNA sequence of its type strain S288c (1). Till date, industrial production of alcohols and other compounds by *S. cerevisiae* has been established.

Currently, biodiesel production has increased worldwide (2). Biodiesel is mainly produced from plant and animal oils (i.e., triacylglycerols) via their transesterification with methanol, neutralization and distillation. Glycerol, generated as one of the by-products during biodiesel production, constitutes up to 10% of the biodiesel produced, and annual glycerol production has reached more than one million tons (3). Glycerol is utilized in the manufacture of cosmetics and food additives, but annual glycerol consumption is more than 750,000 tons, which indicates that the supply exceeds its consumption (4). Therefore, other ways of utilizing glycerol are highly desired. In particular, bioproduction using glycerol as the raw material has been extensively studied as glycerol can be utilized as a growth substrate for microbial cells (4). For example, 1,3-propanediol production by *Clostridium acetobutylicum* (5) and citric acid production by *Yarrowia lipolytica* (6) have been reported.

It is realized that various knowledge of bioproduction using *S. cerevisiae* has been obtained until now. Therefore, to expand availability of *S. cerevisiae* as a bioproduction host, its glycerol assimilation ability has to be improved. In addition, the use of glycerol as the raw material can lead to avoidance of the Crabtree effect in *S. cerevisiae* and can promote efficient bioproduction with reduced formation of ethanol as the by-product.

S. cerevisiae possesses the metabolic pathways for glycerol assimilation (Fig. S1) (7). Glycerol is incorporated into cells via a glycerol/H^+ symporter coded by *STL1* (8) and then converted to glycerol-3-phosphate by glycerol kinase coded by *GUT1* (9). Glycerol-3-phosphate is further converted to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase in the mitochondrial membrane, which is encoded by *GUT2* (10), and then further metabolized via glycolysis and gluconeogenesis. In addition, *S. cerevisiae* has other glycerol assimilation pathways; glycerol is converted to dihydroxyacetone (DHA) by glycerol dehydrogenase coded by *GCY1*, and DHA is converted to DHAP by dihydroxyacetone

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kinase coded by *DAK1* and *DAK2* (11). DHAP is further metabolized via glycolytic and gluconeogenetic pathways. However, *S. cerevisiae* cannot grow well on glycerol as a carbon source and requires other nutrients such as amino acids and nucleic acids for its growth (12). Recently, Klein et al. (13) reported that expression of glycerol facilitator proteins from various yeast species can improve growth of *S. cerevisiae* on glycerol.

It is well known that adaptive laboratory evolution is a powerful tool for understanding the process of evolution. During adaptive laboratory evolution, in which serial transfer of cell culture is performed under specific culture conditions, cells can adapt to perturbations such as changes in the culture environment and modifications in gene expression, and can grow under the altered conditions (14). The recent development of genome-wide analysis systems such as sequencing, and proteomic and metabolomic analyses assists in understanding the mechanisms involved in the adaptive evolution of cells. Recently, adaptive laboratory evolution has been applied to metabolic engineering to obtain strains that are useful for industrial production, for example, strains that are tolerant to stress or exhibit improved ability for resource utilization and/or target material production [for review see Conrad et al. (14) and Dragosits and Mattanovich (15)].

In this study, we used adaptive laboratory evolution experiments and transcriptomic analysis of the evolved strains using RNA sequencing to identify the metabolic engineering targets and improve the glycerol assimilation ability of *S. cerevisiae*. Briefly, we performed serial transfers of the culture of *S. cerevisiae* on glycerol as the main carbon source and observed increase in the specific growth rate of the evolved cells. Subsequently, the transcriptome data of the evolved strains grown on glycerol and glucose were analyzed to identify the target genes for improving the glycerol assimilation ability via metabolic engineering. Finally, we engineered *S. cerevisiae* based on the transcriptome data of the evolved cells and evaluated the identified targets by observing the growth of the engineered strains on glycerol as the main carbon source. In this study, we identified novel metabolic engineering targets for improving glycerol assimilation ability and cell growth on glycerol as the main carbon source of *S. cerevisiae*.

MATERIALS AND METHODS

Strains and media *S. cerevisiae* strain W303-1B (*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*), which was provided by the National Bio-Resource Project, Japan, was used throughout this study. For recombinant DNA experiments, we used *Escherichia coli* DH5α [F⁻ Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA*1 *endA*1 *hsdR*17 (r_K^-, m_K^+) *phoA supE*44 λ⁻ *thi*-1 *gyrA*96 *relA*1], which was purchased from Takara Bio, Inc. (Shiga, Japan).

For cultivating *S. cerevisiae*, MG medium consisting of 0.67% yeast nitrogen base without amino acid (Becton Dickinson Microbiology Systems, Madison, WI, USA), 1% glycerol, 0.02% adenine hemisulfate, and 0.0076% tryptophan, and MD medium in which 1% glycerol was replaced with 2% glucose, were used. When required, 0.038% leucine, 0.076% uracil, and 0.0076% histidine were added to the media. For transformation of *S. cerevisiae*, cells were cultivated in 2 × YPAD medium consisting of 2% Bacto yeast extract (Becton Dickinson Microbiology Systems), 4% Bacto peptone (Becton Dickinson Microbiology Systems), 4% glucose, and 0.04% adenine hemisulfate. In addition, SD media prepared by adding 0.14% yeast synthetic drop-out medium supplements without histidine, leucine, tryptophan, and uracil (Sigma−Aldrich Co. LLC, St. Louis, MO, USA) to the MD media was used for selection of transformants. When preparing plate media for *S. cerevisiae*, 2% agar was added to the medium.

For cultivating *E. coli*, we used the Lennox (L) medium consisting of 1% hipolypepton (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 0.5% dried yeast extract (Nihon Pharmaceutical Co., Ltd.), 0.5% NaCl, and 0.1% glucose (pH 7). When required, 50 μg/mL ampicillin was added to the medium. For preparing L agar plate, 1.5% agar was added to the L medium.

Adaptive laboratory evolution of *S. cerevisiae* on glycerol A single colony of the W303-1B strain grown on MD agar plate was inoculated in 5 mL MD medium and incubated at 30°C for 1 day. After washing the precultured cells with sterilized water, the cells were diluted with 5 mL MG medium to achieve optical density at 660 nm (OD_{660}) of 0.2 and incubated at 30°C. When the OD_{660} of the culture reached around 1, the culture was diluted with 5 mL fresh MG medium to obtain OD_{660} of 0.05 and incubated further at 30°C. This serial transfer process was repeated until the specific cell growth rate increased 2−3 fold compared to the initial specific growth rate. For further analyses, the evolved culture, which was mixed with an equal volume of 30% glycerol, was stored at −80°C at each serial transfer step.

RNA-sequencing For RNA-sequencing, W303-1B and the evolved strains were grown on MD and MG media and total RNA were obtained from the grown cells using the RNeasy mini kit (Qiagen N. V., Venlo, Netherlands).

RNA-sequencing was performed using HiSeq 2500 (Illumina, Inc., San Diego, CA, USA) with 100-bp paired-end reads (100 × 2). The barcode sequences in raw sequence data were trimmed and the trimmed sequence data were mapped to the genome sequence of *S. cerevisiae* S288c. Gene expression data was obtained as values of reads per kilobase of exon per million mapped sequence reads (RPKM). Data processing was performed using CLC Genomics Workbench 8.5.1. (Qiagen N. V.). The summary of the RNA-sequencing analysis and RPKM values for the genes in the samples examined are shown in Tables S1 and S2, respectively.

For principal component analysis (PCA) of the transcriptome data obtained, a free software R (https://www.r-project.org/) (16) was used. In addition, for gene ontology (GO) enrichment analysis, the GO term enrichment tool (http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment) (17) was used. To visualize the expression data of genes related to metabolism, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and KEGG mapper (http://www.genome.jp/kegg/tool/map_pathway2.html) (18). For analyzing the transcription factor binding site, the Find TFs Binding Site(s) tool in Yeastract (http://www.yeastract.com) (19,20) was used and 300-bp upstream sequences from the start codon of the open reading frame was applied to this tool.

Overexpression of *STL1* in *S. cerevisiae* For overexpression of *STL1* in *S. cerevisiae*, the gene fragment was amplified from the *S. cerevisiae* genomic DNA using polymerase chain reaction (PCR) with KOD plus Neo DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) and a set of primers, 5′-GTCGACGACCATGAAGGATTTAAAATTATCG-3′ and 5′-AGATCTATCAACCCTCAAAATTTGCTTTATC-3′ (restriction sites are underlined), and cloned into the T-vector pMD20 (Takara Bio, Inc.). After confirming the sequence of the cloned DNA fragment, the gene was subcloned into the expression vector pGK425 (21). The resulting plasmid, namely pGK425_STL1, as well as an empty vector pGK425, were introduced into *S. cerevisiae* using the lithium acetate method (22).

Overexpression of *HAP4* in *S. cerevisiae* For overexpression of *HAP4*, which encodes a subunit of the transcription factor Hap2p/3p/4p/5p complex, the *HAP4* gene fragment was amplified from the *S. cerevisiae* genomic DNA using the following set of primers: 5′-GTCGACTGGGTTGCTGATTTGTTTTACCTAC-3′ and 5′-GGATCCCGTTTTATTGCAACATGCCTATTTC-3′ (restriction sites are underlined). The methods for cloning *HAP4* into the expression vector pGK426 (21) were identical to those used for *STL1*. The resulting plasmid pGK426_HAP4 was introduced into *S. cerevisiae*.

Disruption of *GIS1* and *RIM15* in *S. cerevisiae* For disrupting *GIS1* and *RIM15*, a *loxP-Sphis5-loxP* gene fragment was amplified using PCR from the pUG27 plasmid (Euroscarf, Frankfurt, Germany) (23) with the following sets of primers: 5′-ATGGAAATCAAGCCAGTTGAGGTTATTGATGGCGTTCCGGTTTTTCAGCTGAAGCTTCGTACGC-3′ and 5′-CTATGATTCAGCTAATTTAGTATTTTCCATTTCTTGAGTGCATGGGCATAGGCCACTAGTGGATCTG-3′ for *GIS1* and 5′-ATGTTCAATAGAAGTAACACCGCAGGCGGATCTCAGGCTATGAAACAGCTGAAGCTTCGTACGC-3′ and 5′-TCAGTGCGTTTCATCAGAATCGCTCAATATAGTATGCTCTTCATCGCATAGGCCACTAGTGGATCTG-3′ for *RIM15*, and each amplified fragment was introduced into *S. cerevisiae* using the lithium acetate method. After obtaining transformants based on complementation of histidine auxotrophy, disruption of each target gene was confirmed by comparing the length of the PCR-amplified fragment from the genome of transformants with that from the genome of the parental strain. For this PCR, primer sets annealing to the upstream and downstream regions of the target gene were used; 5′-CTACAAAGTGAACAACATCG-3′ and 5′-AGGATTCAGAACAGGCAAAG-3′ for *GIS1* and 5′-GAATTCCTAACTCATCAAGC-3′ and 5′-CTTCCGTTTTAATGAACGGG-3′ for *RIM15*.

Measurement of cell growth Cell growth was monitored by measuring OD_{660} using miniphoto518R (Taitec Corporation, Saitama, Japan) and UVmini-1240 (Shimadzu Co., Kyoto, Japan).

RESULTS

Adaptive laboratory evolution of *S. cerevisiae* on glycerol As described in the Introduction, *S. cerevisiae* possesses glycerol catabolic metabolism (Fig. S1). Therefore, we attempted to culture *S. cerevisiae* W303-1B cells on glycerol as the main carbon source without addition of nutrients except for those related to the auxotophy (i.e., MG medium). Although the W303-1B strain did not grow on MG medium for more than

360 h, it started growing at approximately 400 h (Fig. 1). Subsequently, we performed adaptive laboratory evolution experiments using *S. cerevisiae* on glycerol as the main carbon source; we diluted the culture with fresh MG medium after the OD_{660} reached around 1, and then sub-cultured it again (Fig. 1). This serial transfer process was repeated until the specific growth rate of the cells increased 2–3 fold. In this study, two adaptive laboratory evolution cultures, namely culture no. 1 and culture no. 2, were independently propagated. The specific growth rate of the cells increased to approximately 0.05 h^{-1}. Finally, the specific growth rate of the evolved cells reached approximately 0.14 h^{-1} after 1174 h and 1164 h of cultivation for culture no. 1 and no. 2, respectively (Fig. 1).

To confirm whether the *S. cerevisiae* cells can adapt and grow on glycerol as the main carbon source, we cultured cells obtained after 3, 35, and 85 generations (at 432, 890, and 1174 h, respectively) for culture no. 1 and after 3, 30, and 70 generations (at 480, 901, and 1164 h, respectively) for culture no. 2 in MG medium. As shown in Fig. S2, cell growth on glycerol increased with increasing generations via adaptive laboratory evolution.

To confirm whether the glycerol assimilation ability of the evolved cells is stably acquired, we assessed the growth of cells in culture no. 1 after 85 generations on glycerol after serial transfers of the culture in glucose medium (i.e., MD medium). As shown in Fig. S3, the length of the lag phase of cells grown on glycerol after serial transfers in glucose medium was longer (approximately 42 h) than that without serial transfer (approximately 30 h). However, the specific growth rate of cells on glycerol after serial transfers in glucose medium (0.13 h^{-1}) was similar to that without growth on glucose (0.13 h^{-1}). These results indicate that the glycerol assimilation ability of the evolved cells was stable.

In adaptive laboratory evolution, the fitness of evolution increased under one condition, whereas it sometimes decreased under other conditions; this phenomenon is called trade-off (24). To confirm whether such trade-off is observed in our cells that evolved under the condition where *S. cerevisiae* cells can grow on glycerol, we assessed the growth of the evolved cells on glucose as the main carbon source. As shown in Fig. S4, the growth of evolved cells in culture no. 1 after 3, 35, and 85 generations (0.26, 0.28, and 0.28 h^{-1}, respectively) was similar to that of the W303-1B strain (0.30 h^{-1}), indicating that trade-off did not occur in the evolved cells.

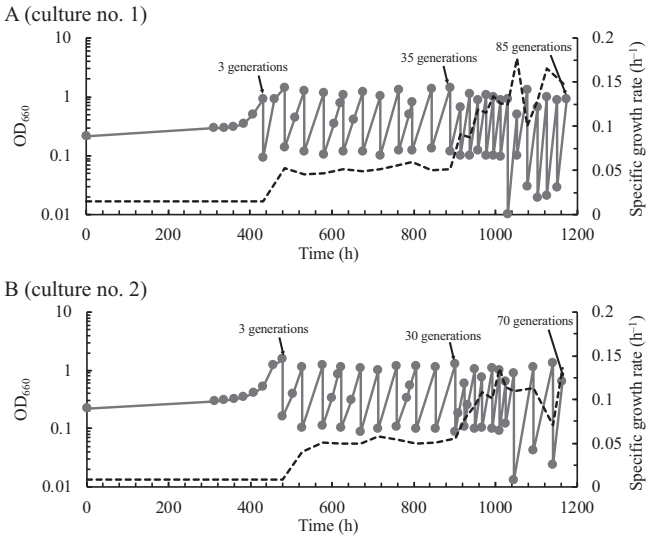

FIG. 1. Adaptive laboratory evolution experiments for *S. cerevisiae* on glycerol as a carbon source. (A) Culture no. 1, (B) culture no. 2. Changes in cell growth (shaded solid line) and specific growth rate (dashed line) through repeated serial transfer of the culture was plotted as a function of cumulative culture time. OD_{660} was measured at the time indicated by closed circle.

Transcriptome analysis of *S. cerevisiae* evolved on glycerol using RNA-sequencing To understand how the *S. cerevisiae* cells adapted to the condition where they could grow well on glycerol as the main carbon source and to identify the metabolic engineering targets for improving glycerol assimilation ability, we performed transcriptomic analysis of the evolved cells obtained from adaptive laboratory evolution; evolved cells grown on glucose or glycerol and the W303-1B strain grown on glucose as the main carbon source (i.e., MG or MD media). In this study, cells obtained after 3, 35, and 85 generations from adaptive laboratory evolution of culture no. 1, and after 3, 30, and 70 generations from adaptive laboratory evolution of culture no. 2 were used for transcriptome analysis. For the transcriptomic analysis, we cultured these strains on glycerol or glucose until cell growth reached the exponential phase and isolated total RNA from the cultured cells. Simultaneously, we also cultured these strains and the parental W303-1B strain on glucose and extracted total RNA from the cultured cells. The isolated RNA samples were subjected to RNA-sequencing analysis (Table S1) and the expression levels of each gene were obtained as RPKM values as described in Materials and methods. In this study, RPKM values for 6903 genes were obtained.

Analysis of the transcriptome data of evolved *S. cerevisiae* on glycerol To understand the process of adaptation of the evolved cells and to identify the engineering targets for improving glycerol assimilation in *S. cerevisiae*, we initially performed PCA using the transcriptome data obtained in this study (Fig. 2). Since RPKM values for 400 genes in all RNA samples examined were zero, we removed RPKM values for these 400 genes and used the RPKM values for the remaining 5903 genes for PCA. The principal components (PCs) 1 and 2 explain 53% and 15% of the variance of transcriptome data, respectively. As shown in the PCA result (Fig. 2), the transcriptome data of the strains cultured on glucose were plotted on the left, whereas those of the strains cultured on glycerol were plotted on the right; PC1 represents the

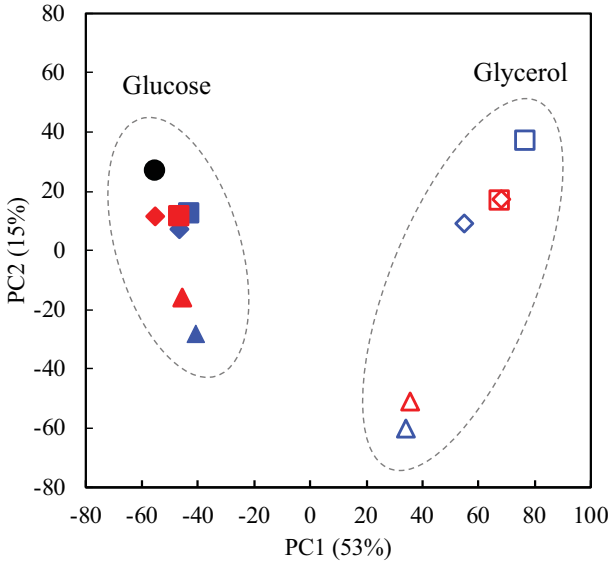

FIG. 2. PCA of transcriptome data of evolved cells grown on glucose and glycerol. Closed and open symbols correspond to the transcriptome data of the cells grown on glucose and glycerol, respectively. Blue square, diamond and triangle represent the transcriptome data for the cells at 3, 35 and 85 generations of the culture no. 1. Red square, diamond and triangle represent the transcriptome data for the cells at 3, 30 and 70 generations of the culture no. 2. Black circle represents the transcriptome data for the W303-1B strain grown on glucose.

TABLE 1. GO term enrichment analysis of the genes with low loadings of PC2 in PCA of transcriptome data.

GO term	*P*-value	Number of genes screened	Number of genes in background[a]
Biological process			
GO:0007005 mitochondrion organization	8.48×10^{-31}	51	391
GO:1902600 hydrogen ion transmembrane transport	1.47×10^{-15}	18	58
GO:0032543 mitochondrial translation	1.52×10^{-14}	23	130
GO:0046034 ATP metabolic process	2.48×10^{-13}	18	75
GO:0009205 purine ribonucleoside triphosphate metabolic process	6.75×10^{-13}	18	79
GO:1901564 organonitrogen compound metabolic process	7.77×10^{-13}	53	1012
GO:0009144 purine nucleoside triphosphate metabolic process	1.09×10^{-12}	18	81
GO:0009199 ribonucleoside triphosphate metabolic process	1.74×10^{-12}	18	83
GO:0098662 inorganic cation transmembrane transport	1.11×10^{-11}	19	106
GO:0009141 nucleoside triphosphate metabolic process	1.20×10^{-11}	18	92
Cellular component			
GO:0044429 mitochondrial part	5.27×10^{-45}	71	601
GO:0005739 mitochondrion	2.30×10^{-39}	82	1107
GO:0098798 mitochondrial protein complex	3.83×10^{-25}	29	105
GO:0098800 inner mitochondrial membrane protein complex	1.29×10^{-24}	26	78
GO:0005743 mitochondrial inner membrane	2.76×10^{-24}	38	243
GO:0044455 mitochondrial membrane part	9.08×10^{-24}	33	169
GO:0005740 mitochondrial envelope	1.00×10^{-23}	46	417
GO:0019866 organelle inner membrane	1.76×10^{-23}	38	255
GO:0031966 mitochondrial membrane	3.20×10^{-22}	43	380
GO:0031975 envelope	7.12×10^{-18}	46	569
Molecular function			
GO:0015078 hydrogen ion transmembrane transporter activity	7.15×10^{-16}	18	56
GO:0015077 monovalent inorganic cation transmembrane transporter activity	2.71×10^{-14}	18	67
GO:0046933 proton-transporting ATP synthase activity, rotational mechanism	5.74×10^{-13}	10	13
GO:0022890 inorganic cation transmembrane transporter activity	3.11×10^{-12}	20	114
GO:0044769 ATPase activity, coupled to transmembrane movement of ions, rotational mechanism	2.12×10^{-8}	10	28
GO:0008324 cation transmembrane transporter activity	6.28×10^{-8}	20	190
GO:0016675 oxidoreductase activity, acting on a heme group of donors	3.73×10^{-7}	7	12
GO:0016676 oxidoreductase activity, acting on a heme group of donors, oxygen as acceptor	3.73×10^{-7}	7	12
GO:0015002 heme-copper terminal oxidase activity	3.73×10^{-7}	7	12
GO:0004129 cytochrome-c oxidase activity	3.73×10^{-7}	7	12

One hundred genes with low loadings of PC2 in PCA was analyzed. Ten GO terms for each category, "Biological process," "Cellular component" and "Molecular function," with the lowest *P*-values are shown.

[a] In background, 5739 genes are included.

transcriptome change caused by the difference in the carbon sources. Furthermore, the transcriptome data of the strains at the early generations in the adaptive laboratory evolution experiments were plotted on the upper panel, whereas those at the late generations were plotted on the lower panel in the figure; PC2 represents the transcriptional changes during adaptation to the environment that enabled growth on glycerol as the carbon source.

To understand the mechanisms of adaptive evolution of *S. cerevisiae* grown on glycerol, we analyzed enrichment of the functional category of genes contributing to the determination of PC2, as PC2 represents the gene expression changes during adaptive evolution on glycerol. We identified 100 genes with the highest and lowest loadings of PC2 and analyzed the GO term enrichment of the identified genes. However, GO terms for genes with high loading of PC2 were not enriched. In contrast, GO terms related to mitochondria function and oxidative phosphorylation were statistically enriched for genes with low loading of PC2 (Table 1). These results indicate that genes related to mitochondria function and oxidative phosphorylation are responsible for adaptation to an environment with glycerol as the carbon source for growth.

Next, we compared the gene expression profile of the evolved cells in culture no. 1 with that of culture no. 2. As shown in Fig. S5A, whole gene expression of the evolved cells in culture no. 1 after 2, 35, and 85 generations was similar to that in culture no. 2 at 3, 30, and 70 generations, respectively. This phenomenon was also observed in case of genes related to the carbon metabolism, including the glycolysis, the TCA cycle, ethanol fermentation, the pentose phosphate pathway, the oxidative phosphorylation, and glycerol metabolism (Fig. S5B). Therefore, we subsequently analyzed the gene expression profile of the evolved cells in culture no. 1.

Analysis of the expression of genes related to carbon metabolism in the evolved cells of *S. cerevisiae* We analyzed changes in the expression of genes related to metabolism in the KEGG database in cells of culture no. 1 after 85 generations of adaptive laboratory evolution and compared it with those of cells after 3 generations. We found that the expression of genes related to the TCA cycle and oxidative phosphorylation was upregulated and that related to the pentose phosphate pathway was downregulated (Fig. S6). Therefore, we analyzed in detail the expression of genes related to the TCA cycle, oxidative phosphorylation, and the pentose phosphate pathway in the adapted cells of culture no. 1 grown on glycerol and in the W303-1B grown on glucose. As shown in Fig. 3, the expression of genes related to the TCA cycle and oxidative phosphorylation in the evolved cells after 3 generations grown on glycerol was higher than that in the W303-

1B strain grown on glucose. In addition, the expression of some genes related to the TCA cycle and oxidative phosphorylation was higher in the cells after 85 generations than in the cells after 3 and 35 generations when growing on glycerol as the main carbon source. These phenomena were consistent with the results obtained using PCA (Fig. 2 and Table 1).

As for the pentose phosphate pathway (Fig. 4), expression of *SOL4*, *GND2*, *TKL2*, and *NQM1* was higher in cells after 3 generations on glycerol than in the W303-1B strain grown on glucose. Among them, the expression of *GND2*, *TKL2*, and *NQM1*, related to the non-oxidative branch of the pentose phosphate pathway, was down-regulated in cells after 85 generations, compared to in cells after 3 and 35 generations.

Among the genes involved in glycerol uptake and assimilation, the expression of *STL1*, *GUT1*, and *GUT2* encoding glycerol/H^+ symporter, glycerol kinase, and glycerol-3-phosphate dehydrogenase, respectively, was upregulated in the evolved cells of culture no. 1 at 3 generations compared to that in the parental W301-1B cells grown on glucose (Fig. S7). Furthermore, the expression of these genes in culture no. 1 were elevated (*GUT1*) or did not change (*STL1* and *GUT2*) in the cells after 35 and 85 generations compared to that in the cells after 3 generations. A similar phenomenon was also observed regarding the expression of genes related to the other glycerol assimilation pathway (*GCY1* and *DAK2*) (Fig. S7). Upregulation of genes related to glycerol uptake and assimilation by changing the carbon source from glucose to glycerol, and

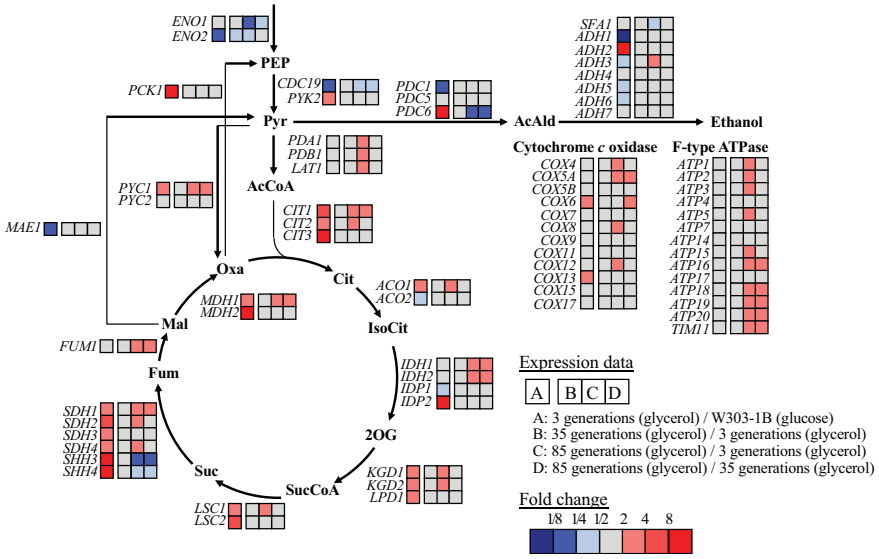

FIG. 3. Expression of the genes for the TCA cycle, oxidative phosphorylation and ethanol formation in the evolved cells obtained from culture no. 1. Abbreviations for the name of metabolite are listed in Table S3.

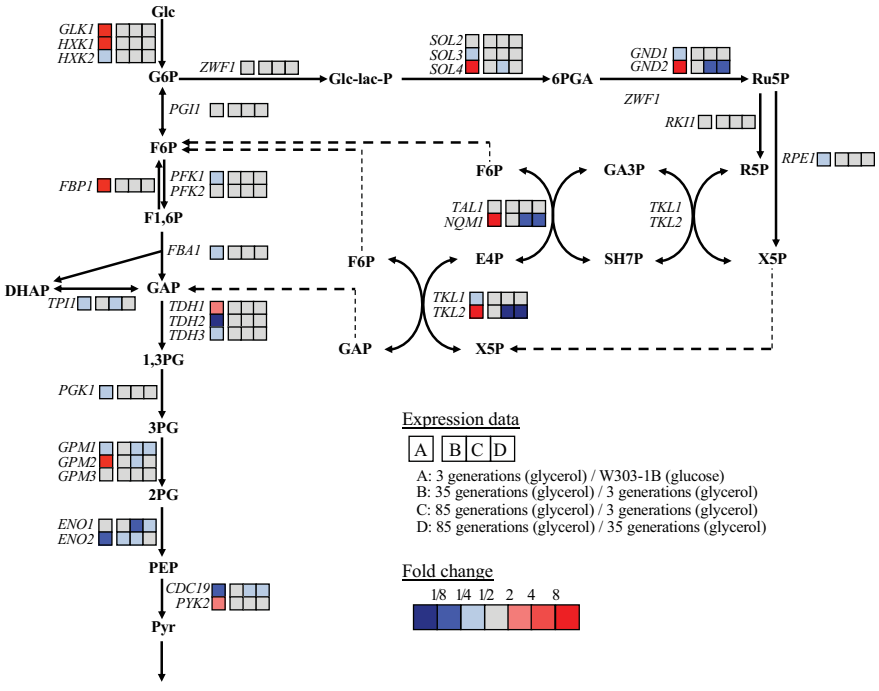

FIG. 4. Expression of the genes for the pentose phosphate pathway in the evolved cells obtained from culture no. 1. Expression of the genes for the glycolysis is also shown.

maintenance of their upregulated expression levels through evolution on glycerol is important for the growth of *S. cerevisiae* on glycerol.

Among the genes involved in the glycolytic and gluconeogenetic pathways (Fig. 4), the expression of *FBP1* encoding fructose bisphosphatase in gluconeogenesis, and that of *GPM2* encoding the glycolytic enzyme phosphoglucomutase, increased dramatically in the evolved cells after 3 generations compared to that in the W303-1B grown on glucose. In addition, the expression of most genes encoding glycolytic enzymes did not change or decreased in the evolved cells after 35 and 85 generations compared to that in the cells after 3 generations when growing on glycerol as the main carbon source.

Identification of engineering targets based on transcriptomic data of evolved cells to improve the glycerol assimilation ability of *S. cerevisiae* Finally, we identified metabolic engineering targets for improving the glycerol assimilation ability of *S. cerevisiae* based on the transcriptome data obtained for the evolved cells and evaluated the identified targets by assessing the growth of the engineered strains on glycerol as the main carbon source.

Transcriptome data analysis indicated that the upregulation of genes related to the TCA cycle and oxidative phosphorylation and downregulation of genes related to the non-oxidative branch of the pentose phosphate pathway might improve glycerol assimilation in *S. cerevisiae*. For the upregulation of genes related to the TCA cycle and oxidative phosphorylation, we examined the effect of overexpression of *HAP4* on growth of *S. cerevisiae* on glycerol. The Hap4p protein is one of the subunits of the transcription activator complex Hap2p/3p/4p/5p (25) and it has been reported that *HAP4* overexpression can upregulate the expression of genes related to the TCA cycle and oxidative phosphorylation (26). Expression of *HAP4* in the evolved cells of culture no. 1 after 85 generations increased 5-fold compared to that in the evolved cells after 3 generations (Table S2). As shown in Fig. 5, the length of the lag phase in the *HAP4*-overexpressing W303-1B/pGK426_HAP4 strain (more than 300 h) was shorter than that in the control W303-1B/pGK426 strain (more than 480 h), although a dramatic reduction in the length of the lag phase was not observed. However, reduction in the length of the lag phase was clearly observed by combining *HAP4* overexpression with *STL1* overexpression; the length of the lag phase in the W303-1B strain transformed with pGK425_STL1 and pGK426_HAP4 (approximately 150 h) was significantly reduced compared to the W303-1B transformed with pGK425 and pGK426_HAP4 (approximately 250 h).

As described above, genes related to the pentose phosphate pathway (i.e., *GND2*, *TKL2*, and *NQM1*) were downregulated in the evolved strain grown on glycerol. To determine the strategy for downregulating these genes, common transcription factor-binding sites in the upstream regions of the *GND2*, *TKL2*, and *NQM1* open reading frames were identified. As a result, we identified the binding site of a transcription factor Gis1p in the upstream region of these three genes (Fig. S8). We, therefore, examined the effect of *GIS1* disruption in *S. cerevisiae* W303-1B on growth on glycerol. *GIS1* encodes a transcriptional activator involved in signal transduction in response to the availability of nutrients, such as TOR, Sch9, and PKA (27–29). We expected that *GIS1* disruption might lead to downregulation of *GND2*, *TKL2*, and *NQM1*. However, the *GIS1*-disrupted strain was not able to grow on glycerol as the main carbon source (Fig. S9).

In addition, *GIS1* transcription is regulated by the Rim15p protein kinase (27), which was originally isolated as a gene responsible for the regulation of meiotic genes (30) and was identified as a component of the Ras/cAMP pathway (31). Hence, we investigated the effect of *RIM15* disruption, which might reduce *GIS1* transcription, on growth of the W303-1B strain on glycerol. As shown in Fig. S9, growth of the *RIM15*-disrupted strain started at approximately 200 h, which was earlier than that of the control W303-1B strain (at approximately 400 h). Furthermore, *STL1* overexpression further reduced the length of lag phase to about 48 h in the *RIM15*-disrupted strain and the specific growth rate of the *RIM15*-disrupted and *STL1*-overexpressing strain reached 0.056 h^{-1} (Fig. 6). These results indicate that *RIM15* disruption improves the glycerol assimilation ability of *S. cerevisiae* and *STL1* overexpression has a synergistic effect on its improvement.

Finally, we investigated the effect of *HAP4* overexpression on glycerol assimilation of *RIM15*-disrupted and *STL1*-overexpressing strain. As shown in Fig. S10, the synergistic effect of *HAP4* overexpression was not observed; the specific growth rate of the *RIM15*-disrupted and *HAP4*- and *STL1*-overexpressing strain during exponential growth (0.061 h^{-1}) was not significantly increased compared to that of *RIM15*-disrupted and *STL1*-overexpressing strain.

DISCUSSION

In this study, we attempted to identify metabolic engineering targets to improve the glycerol assimilation ability of *S. cerevisiae* using adaptive laboratory evolution. Based on the transcriptome data of cells obtained using adaptive laboratory evolution on glycerol, we identified the engineering targets for improving the

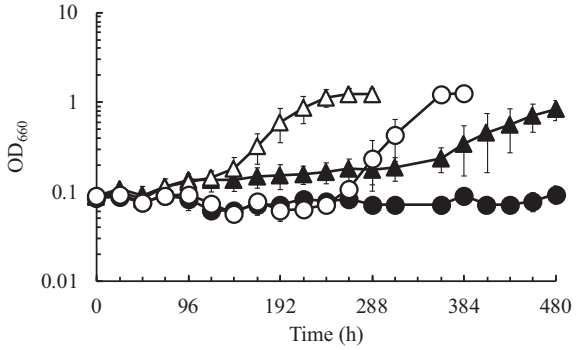

FIG. 5. Effect of overexpression of *HAP4* gene on growth of the W303-1B strain on glycerol as a main carbon source. Cell growth of the *HAP4*- and/or *STL1*-overexpressing W303-1B strain is shown. The average ±standard deviation in multiple independent experiments is shown; triplicates for the W303-1B strain transformed with pGK425 and pGK426 (closed circles), 4 replicates for the W303-1B strain transformed with pGK425 and pGK426_HAP4 (closed triangles), triplicates for the W303-1B strain transformed with pGK425_STL1 and pGK426 (open circles), and 5 replicates for the W303-1B strain transformed with pGK425_STL1 and pGK426_HAP4 (open triangles).

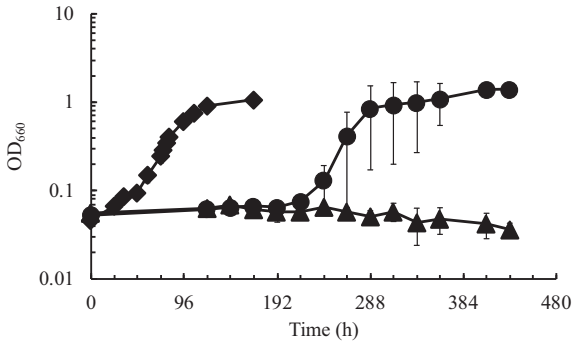

FIG. 6. Effect of disruption of *GIS1* and *RIM15* genes on growth of the *STL1*-overexpressing W303-1B strain on glycerol as a main carbon source. Cell growth of the control (circles), *GIS1*-disrupted (triangles), *RIM15*-disrupted (diamonds) strains is shown. The average ± standard deviation in 3 independent experiments is shown.

glycerol assimilation ability of *S. cerevisiae*, which was indeed accomplished by overexpressing *HAP4* or disrupting *RIM15* along with *STL1* overexpression.

As glycerol is known to be one of the non-fermentable carbon sources for *S. cerevisiae*, we expected that upregulation of the TCA cycle and oxidative phosphorylation by *HAP4* overexpression would improve its glycerol assimilation ability. Recently, Olivares-Marin et al. (32) reported the dependence of nutrition homeostasis on *RIM15*; the *RIM15*-disrupted strain showed severe growth defect on non-fermentable carbon sources such as glycerol, and disruption of *RIM15* improved fermentation ability by inhibiting respiration capacity. This inconsistency between the results might be caused by differences in the media; in that study, a medium consisting of yeast extract, peptone, and glycerol was used, whereas a synthetic medium (i.e., MG medium) was used in our study.

Although the mechanism responsible for the downregulation of genes related to the pentose phosphate pathway are not known, this might be related to reduction of NADPH formation. As described in Introduction, *S. cerevisiae* possesses two glycerol assimilation pathways; one of them consists of Gut1p and Gut2p, whereas the other consists of Gcy1p, Dak1p, and Dak2p (Fig. S1). Similar to expression of the *GUT1* and *GUT2*, expression of *GCY1* and *DAK2* was upregulated in the evolved cells in culture no. 1 after 3 generations grown on glycerol as the main carbon source compared to the W303-1B strain grown on glucose. However, the expression remained unchanged in the evolved cells after 35 and 85 generations compared to that in the evolved cells after 3 generations (Fig. S7). Furthermore, this was similar to the expression of *GND2*, *TKL2*, and *NQM1* as described in Results. If the pathway consisting of Gcy1p and Dak2p is used for glycerol assimilation in the evolved cells, NADPH is produced via the reaction catalyzed by Gcy1p. Therefore, to reduce NADPH formation in other metabolic pathways, genes related to the pentose phosphate pathway might be downregulated, and as a result, the metabolic flux through the pentose phosphate pathway might be reduced (Fig. 4). Another possibility is that changes in the expression of other genes, whose regulation is similar to that for *GND2*, *TKL2*, and *NQM1*, appears to be more important for adaptation to the environment where *S. cerevisiae* cells can grow on glycerol, than the downregulation of the pentose phosphate pathway genes. Further investigations on the relation between expression changes in the pentose phosphate pathway genes and glycerol assimilation ability are required.

Studies on adaptive laboratory evolution of *S. cerevisiae* grown on glycerol have been reported. Merico et al. (33) showed that the specific growth rate of the evolved strain on glycerol medium containing peptone reached 0.110 h^{-1} for the CEN.PK 113-7D strain and 0.036 h^{-1} for the W303 strain and the evolved strains, both of which were lower than that observed in our evolution experiment. The evolved strains exhibited ethanol productivity similar to that of the original cells and were freeze-tolerant. Ochoa-Estopier et al. (34) reported that the specific growth rate of the evolved strain on glycerol reached 0.2 h^{-1}. In addition, the specific growth rate of the evolved cells on glycerol obtained in this study (0.14 h^{-1}) was higher than that of the evolved cells reported by Ho et al. (35); the specific growth rates of the evolved strains of the CEN.PK113-1A, namely PW-1 and PW-2, reached 0.119 h^{-1} and 0.127 h^{-1}, respectively. Ho et al. (35) also reported the effect of mutations in the evolved strains on growth on glycerol, suggesting that introduction of two point mutations in *UBR2* and *GUT1* increased the specific growth rate to 0.130 h^{-1}. In contrast, the specific growth rate of the evolved cells obtained in our experiments was lower than that of the evolved strain reported by Strucko et al. (36); the specific growth rate of the evolved strain derived from the CEN.PK113-7D was 0.22 h^{-1}, and a triple mutant strain harboring mutations in *GUT1*, *KGD1*, and *UBC13* identified by genome resequencing of the evolved strain restored the growth phenotype of the evolved strain after about 300 generations and its specific growth rate was more than 0.2 h^{-1}. The specific growth rate of the recombinant strain engineered based on the transcriptome data of the evolved strain in this study was lower than those of the evolved cells and recombinant strain constructed based on adaptive laboratory evolution in previous studies. However, we identified novel genes contributing to growth on glycerol as the main carbon source, such as *HAP4* and *RIM15*, based on the transcriptome analysis of the evolved strains in this study. Analysis of the relationship between the previously identified genes and *HAP4* and *RIM15* would be important for deciphering glycerol metabolism and further improving glycerol assimilation ability.

However, the lag phases of the *HAP4*- and *STL1*-overexpressing strain (approximately 150 h) (Fig. 5) and the *RIM15*-disrupted and *STL1*-overexpressing strain (approximately 48 h) (Fig. 6) were longer than those of the evolved cells in culture no. 1 after 85 generations (approximately 12 h) (Fig. S2). In addition, the specific growth rates of the *HAP4*- and *STL1*-overexpressing strain (0.024 h^{-1}) (Fig. 5) and the *RIM15*-disrupted and *STL1*-overexpressing strain (0.056 h^{-1}) (Fig. 6) during exponential growth was lower than that of evolved cells of culture no. 1 after 85 generations (0.14 h^{-1}) (Fig. S2). These observations suggest that other factors contributing to glycerol assimilation are present in the evolved cells and further studies are necessary for identifying the targets required for improving growth on glycerol as the carbon source. As shown in Fig. S3, the glycerol assimilation ability of the evolved cells was stably maintained and this phenomenon is possibly caused by mutations in the genome. Further studies, particularly genome resequencing of the evolved cells obtained in this study, will be effective for identifying the target genes to improve growth on glycerol as the carbon source.

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