Improvement of Glucose Uptake Rate and Production of Target Chemicals by Overexpressing Hexose Transporters and Transcriptional Activator Gcr1 in *Saccharomyces cerevisiae*

Daehee Kim, Ji-Yoon Song,* Ji-Sook Hahn

School of Chemical and Biological Engineering, Seoul National University, Seoul, Republic of Korea

Metabolic engineering to increase the glucose uptake rate might be beneficial to improve microbial production of various fuels and chemicals. In this study, we enhanced the glucose uptake rate in *Saccharomyces cerevisiae* by overexpressing hexose transporters (HXTs). Among the 5 tested HXTs (Hxt1, Hxt2, Hxt3, Hxt4, and Hxt7), overexpression of high-affinity transporter Hxt7 was the most effective in increasing the glucose uptake rate, followed by moderate-affinity transporters Hxt2 and Hxt4. Deletion of *STD1* and *MTH1*, encoding corepressors of *HXT* genes, exerted differential effects on the glucose uptake rate, depending on the culture conditions. In addition, improved cell growth and glucose uptake rates could be achieved by overexpression of *GCR1*, which led to increased transcription levels of *HXT1* and ribosomal protein genes. All genetic modifications enhancing the glucose uptake rate also increased the ethanol production rate in wild-type *S. cerevisiae*. Furthermore, the growth-promoting effect of *GCR1* overexpression was successfully applied to lactic acid production in an engineered lactic acid-producing strain, resulting in a significant improvement of productivity and titers of lactic acid production under acidic fermentation conditions.

*S*accharomyces cerevisiae has been used as an important cell factory for production of fuels and chemicals (1, 2). Although yeast can utilize a wide range of carbon sources, glucose is the most widely preferred carbon source for yeast fermentation (3). Glucose uptake in *S. cerevisiae* is elaborately regulated to adapt cellular metabolism in response to ever-changing environmental conditions (3, 4). However, to achieve high titers and productivity of target chemicals using metabolically engineered yeast cells, it may be beneficial to inactivate, circumvent, or modify some of such natural regulatory mechanisms.

The first regulatory step of glucose uptake is the facilitated diffusion of glucose through hexose transporters (HXTs) in the plasma membrane (4, 5). *S. cerevisiae* contains 18 HXT family members of hexose transporters, Hxt1 to Hxt17 and Gal2, among which Hxt1 to Hxt7 function as major glucose transporters (6). Expression of the HXTs, all of which exhibit different levels of glucose affinity, is differentially regulated depending on extracellular glucose concentrations (7–9). Hxt1 ($K_m = {\sim}100$ mM) and Hxt3 ($K_m = {\sim}30$ to 60 mM) are classified as low-affinity glucose transporters. *HXT1* is highly induced in the presence of high (>1%) concentrations of glucose, whereas *HXT3* is induced by both low and high levels of glucose. When the glucose concentrations are lowered to around 0.1%, *HXT2* and *HXT4*, encoding moderate-affinity glucose transporters ($K_m = {\sim}5$ to 10 mM), are expressed (10). *HXT6* and *HXT7*, encoding highly homologous glucose transporters with very high glucose affinity ($K_m = {\sim}1$ mM), are expressed just before the depletion of glucose in the medium (11). Expression of *HXT5*, encoding a moderate-affinity transporter ($K_m = {\sim}10$ mM), is not regulated by extracellular glucose concentrations but by various environmental conditions affecting growth rates (12, 13).

The glucose concentration-dependent expression of *HXT* genes is achieved by a complex interconnection of both glucose induction and glucose repression mechanisms (14, 15). Induction of the *HXT1*, *HXT2*, *HXT3*, and *HXT4* genes in the presence of glucose is mainly regulated by plasma membrane glucose sensors Snf3 and

Rgt2 and by a transcriptional repressor, Rgt1 (16, 17). In the absence of glucose, *HXT* genes are repressed by Rgt1 in association with corepressors Mth1 and Std1 (18, 19). In the presence of glucose, binding of glucose to Snf3 and Rgt2 triggers a signal corresponding to the degradation of Mth1 and Std1, resulting in the release of Rgt1 from the promoters and subsequent derepression of *HXT* genes (20). On the other hand, *HXT2*, *HXT3*, *HXT4*, *HXT6*, and *HXT7* are repressed by Mig1 in the presence of glucose (10, 21). Upon glucose starvation, activated Snf1 kinase phosphorylates Mig1, leading to its nuclear export and derepression of its target genes.

Previously, it was shown that overexpression of *HXT1* or *HXT7* in *S. cerevisiae* increases the rates of glucose uptake and ethanol production (22). Moreover, overexpression of *HXT1* or *HXT7* led to increased productivity and titers of lactic acid in *S. cerevisiae* cells engineered for lactic acid production, suggesting the possibility of manipulating the glucose uptake rate to improve fermentation performance for the production of various target chemicals under conditions in which optimal metabolic pathways toward the target product are provided (22). In addition to an increase in HXT protein levels in the plasma membrane, an increase in glycolytic flux might serve as a driving force to enhance the glucose uptake rate. However, so far, overexpression of glycolytic enzymes indi-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
S. cerevisiae CEN.PK2-1C	*MAT***a** *ura3-52 trp1-289 leu2-3_112 his3Δ1 MAL2-8c SUC2*	EUROSCARF[a]
S. cerevisiae JHY701	CEN.PK2-1C *mth1Δ*	This study
S. cerevisiae JHY702	CEN.PK2-1C *std1Δ*	This study
S. cerevisiae JHY703	CEN.PK2-1C *mth1Δ std1Δ*	This study
S. cerevisiae SP2006	CEN.PK2-1C *pdc1Δ*::P_{ccw12}-*BtLDH cyb2Δ*::P_{ccw12}-*BtLDH gpd1Δ*::P_{ccw12}-*PsjLDH adh1Δ*::P_{ccw12}-*PsjLDH*	29
S. cerevisiae SP2018	SP2006 *his3Δ*::P_{TDH3}-*GCR1*	This study
S. cerevisiae SP1130	SP2006 *ald6Δ leu2Δ*::P_{TDH3}-*mhpF pdc6Δ*::P_{TDH3}-*eutE*	29
S. cerevisiae SP1141	SP1130 *his3Δ*::P_{TDH3}-*GCR1*	This study
Plasmids		
pRS414GPD	CEN/ARS plasmid, P_{TDH3}, T_{CYC1}, *TRP1* marker	30
pRS415GPD	CEN/ARS plasmid P_{TDH3}, T_{CYC1}, *LEU2* marker	30
pRS416GPD	CEN/ARS plasmid, P_{TDH3}, T_{CYC1}, *URA3* marker	30
pUG72	Plasmid containing *loxP-URA3-loxP* deletion cassette	EUROSCARF
pSH63	Plasmid containing a gene for Cre-recombinase, *TRP1* marker	EUROSCARF
pRS414GPD-*HXT1*	*HXT1* ORF cloned between the BamHI and ClaI sites of pRS414GPD	This study
pRS414GPD-*HXT2*	*HXT2* ORF cloned between the BamHI and ClaI sites of pRS414GPD	This study
pRS414GPD-*HXT3*	*HXT3* ORF cloned between the BamHI and ClaI sites of pRS414GPD	This study
pRS414GPD-*HXT4*	*HXT4* ORF cloned between the BamHI and ClaI sites of pRS414GPD	This study
pRS414GPD-*HXT7*	*HXT7* ORF cloned between the BamHI and XhoI sites of pRS414GPD	This study
pRS416GPD-*GCR1*	*GCR1* ORF cloned between the XbaI and XhoI sites of pRS415GPD	This study
pRS415GPD-*GCR2*	*GCR2* ORF cloned between the XbaI and XhoI sites of pRS416GPD	This study
pUC57-URA3	Plasmid containing *HA-URA3-HA* deletion cassette	29
pUC57-URA3–*GCR1*	P_{TDH3}-*GCR1*-T_{CYC1} cloned into the KpnI site of pUC57-URA3	This study

[a] EUROSCARF, European *Saccharomyces cerevisiae* Archive for Functional Analysis.

vidually or in combination in experiments designed to significantly improve glycolytic flux has not been successful (23–27). In general, the highest yield of a desired chemical can be reached by maximizing the metabolic flux to the desired product while minimizing biomass synthesis. However, the drain of metabolites from essential pathways and/or product toxicity could inhibit cell growth, exerting a negative effect on the production of the target chemical by decreasing volumetric productivity. Therefore, a proper balance between growth rate and production flux is important to achieve both high yield and productivity (28).

In this study, we improved the glucose uptake rate in *S. cerevisiae* by overexpressing HXTs with various glucose affinities. In addition, improved cell growth and glucose uptake rates could be achieved by overexpressing a transcriptional activator, Gcr1, which led to increased expression levels of *HXT1* and genes encoding ribosomal proteins (RPs). Overexpression of *GCR1* was successfully applied to lactic acid production in an engineered strain, resulting in a significant improvement of lactic acid production under acidic fermentation conditions, where overcoming the growth-inhibitory effect is critical to improve lactic acid production.

MATERIALS AND METHODS

Yeast strains, media, and culture conditions. The *S. cerevisiae* strains used in this study and the primers used for the construction of the strains are described in Table 1 and Table 2, respectively. All *S. cerevisiae* strains used in this study were derivatives of CEN.PK2-1C. JHY701 (*mth1Δ*), JHY702 (*std1Δ*), and JHY703 (*mth1Δ std1Δ*) strains were generated by deleting *MTH1* and/or *STD1* by PCR-mediated homologous recombination based on the Cre/loxP recombination system (31). The integration cassettes for the deletion of *MTH1* and *STD1* were obtained by PCR am-

plification from pUG72, using primer pair d_*MTH1* F and d_ *MTH1* R or primer pair d_*STD1* F and d_ *STD1* R. After confirmation of the correct integration of the cassette by PCR analysis using primer pairs consisting of ch_*MTH1* F or ch_*STD1* F with Ura-B and ch_*MTH1* R or ch_*STD1* R with Ura-C for each gene, the marker gene was removed by transformation with Cre recombinase expression vector pSH63. Strain JHY703 was generated by additional deletion of the *STD1* gene in strain JHY701 by using the same method. Lactic acid-producing strains SP2006 and SP1130, possessing lactate dehydrogenase (*LDH*) genes originating from *Pelodiscus sinensis* subspecies *japonicus* (*PsjLDH*) or *Bos taurus* (*BtLDH*), were described previously (29). In CEN.PK2-1C, open reading frames (ORFs) of *ADH1*, *PDC1*, *GPD1*, and *CYB2* genes were replaced with either the *BtLDH* gene or the *PsjLDH* gene controlled by the *CCW12* promoter, resulting in strain SP2006 (Table 1) (29). To generate strain SP1130 (Table 1), P_{TDH3}-controlled *Escherichia coli mhpF* and *eutE* genes, encoding acetylating acetaldehyde dehydrogenase (A-ALD), were introduced into the chromosome of SP2006, and *ALD6* was additionally deleted (29). To generate strains SP2018 and SP1141 (overexpressing *GCR1*), the P_{TDH3}-*GCR1*/*HA-URA3-HA* cassette was PCR amplified using primers i_*GCR1* F and i_*GCR1* R from plasmid pUC57-URA3-*GCR1* and integrated into the chromosomes of SP2006 and SP1130, respectively, by replacing *his3Δ1*. After confirmation of the correct integration of the cassette by PCR analysis using primer pair ch_*HIS3* F and ch_*GCR1* R, the *URA3* marker flanked with hemagglutinin (HA) tags was popped out (32). CEN.PK2-1C yeast cells were precultured in yeast extract-peptone-dextrose (YPD; 1% yeast extract, 2% Bacto peptone, and dextrose) medium or synthetic complete (SC; 0.67% yeast nitrogen base without amino acids and 0.2% amino acid dropout mixture lacking appropriate components for selection) medium containing 4% glucose and were inoculated to an A_{600} of 5 in YPD or SC medium containing 8% glucose. Cells in a 5-ml volume were cultivated in 50-ml screw-cap conical tubes at 30°C with shaking at 170 rpm. For lactic acid fermentation, lactic acid-producing strains were inoculated from an overnight seed culture at an A_{600} of 0.5

TABLE 2 Primers used in this study

Primer and category	Sequence[a]
Strain construction	
d_*MTH1* F	5′-GAATTTTATTCGAACGCATAGAGTACACACACTCAAAGGACAGCTGAAGCTTCGTACGC-3′
d_*MTH1* R	5′-TCCAAAAAAACCATCGGGAAGGTTTCTTTTTAGTATCTGCATAGGCCACTAGTGGATCTG-3′
d_*STD1* F	5′-GTAGGAGGTTTTGCACTACTTAACAGACAAATAAAACGAGCAGCTGAAGCTTCGTACGC-3′
d_*STD1* R	5′-AGGACATTCCATCAGGCTTCCAATCTAACCCCAACCTTGCATAGGCCACTAGTGGATCTG-3′
i_*GCR1* F	5′-TATACTAAAAAATGAGCAGGCAAGATAAACGAAGGCAAAGGGTTTCCCGACTGGAAAGC-3′
i_*GCR1* R	5′-ATATATCGATTGCTGCAGCTTTAAATAATCGGTGTCACTACCAGTCACGACGTTGTAAAA-3′
ch_*MTH1* F	5′-TTCCTTTCTTCTCAAACTTT-3′
ch_*MTH1* R	5′-TCTTTACTGCGATAACGGT-3′
ch_*STD1* F	5′-GTTCCATAACGGAAAAATT-3′
ch_*STD1* R	5′-ACTTAATCTTAAAGGTCGT-3′
ch_*HIS3* F	5′-ATGCCTCGGTAATGATTTTC-3
ch_*GCR1* R	5′-CCGCTAGTAGCGTAATCTG-3′
Ura-B	5′-CAGACCGATCTTCTACCC-3′
Ura-C	5′-TTGGCTAATCATGACCCC-3′
Plasmid construction	
HXT1 F	5′-CGC*GGATCC*ATGAATTCAACTCCCG-3′
HXT1 R	5′-CGC*ATCGAT*TTATTTCCTGCTAAACAAAC-3′
HXT2 F	5′-CGC*GGATCC*ATGTCTGAATTCGCTACTAG-3′
HXT2 R	5′-CGC*ATCGAT*TTATTCCTCGGAAACTCT-3′
HXT3 F	5′-CGC*GGATCC*ATGAATTCAACTCCAGATTT-3′
HXT3 R	5′-CGC*ATCGAT*TTATTTCTTGCCGAACAT-3′
HXT4 F	5′-CGC*GGATCC*ATGTCTGAAGAAGCTGC-3′
HXT4 R	5′-CGC*ATCGAT*CTACTTTTTTCCGAACATC-3′
HXT7 F	5′-CGC*GGATCC*ATGTCACAAGACGCTG-3′
HXT7 R	5′-CGC*CTCGAG*TTATTTGGTGCTGAACA-3′
GCR1 F	5′-CGC*TCTAGA*ATGGTATGGTATGATCATAACA-3′
GCR1 R	5′-CGC*CTCGAG*TTAAGATGGTGTATTATGTCG-3′
GCR2 F	5′-CGC*TCTAGA*ATGCATCACCAAACTAAGT-3′
GCR2 R	5′-CGC*CTCGAG*TCATCTTTGTAAATCCCTTAA-3′
c_*GCR1* F	5′-CGC*GGTACC*TCATTATCAATACTCGCCATTTCA-3′
c_*GCR1* R	5′-CCC*GGTACC*CAAATTAAAGCCTTCGAGCG-3′
qRT-PCR	
p_*HXT1* F	5′-CCATTAAGAGTCAAATCC-3′
p_*HXT1* R	5′-GTAAGCGAAAACCATACA-3′
p_*HXT2* F	5′-TCTTCTTCGCTATTAGTT-3′
p_*HXT2* R	5′-TTGTAATGAAGGGAGTGA-3′
p_*HXT3* F	5′-TTGTGGGGTTTCTTGATT-3′
p_*HXT3* R	5′-TTCTTCCAAAGTCAAACC-3′
p_*HXT4* F	5′-ATGGCTATTGCTCAAGCT-3′
p_*HXT4* R	5′-TGGAACGAAGAAGAAGAC-3′
p_*ADH1* F	5′-TTATTCAGATCCATCGGT-3′
p_*ADH1* R	5′-GTATCTGGTAGAAGCTTC-3′
p_*ENO1* F	5′-CAACCCAAAGAGAATTGC-3′
p_*ENO1* R	5′-AGATCTGTGGGAAACCAT-3′
p_*GPM1* F	5′-GAATCTTTGGCTTTGGTC-3′
p_*GPM1* R	5′-AGCATCAGAGATACCTTC-3′
p_*PGK1* F	5′-CAATTGATTGACAACTTG-3′
p_*PGK1* R	5′-TTCCATCAACTTTGGAAC-3′
p_*RPL11A* F	5′-CGGTATTGACGAACACATTGACTTGG-3′
p_*RPL11A* R	5′-TACCCTTACATCTCTTTCTTCTAGTG-3′
p_*RPS18B* F	5′-TCAAAACGACATTACTGATGGTAAGG-3′
p_*RPS18B* R	5′-CCAGAAGTGTCTAATACCACGGTGG-3′
p_*RPL30* F	5′-CATCATTGCCGCTAACACTCCAGTTTTG-3′
p_*RPL30* R	5′-CCAGCTTCCAAAATAGAGACAACACCG-3′
p_*ACT1* F	5′-GCCGAAAGAATGCAAAAGGA-3′
p_*ACT1* R	5′-TAGAACCACCAATCCAGACGG-3′

[a] Utilized restriction sites are shown in italics.
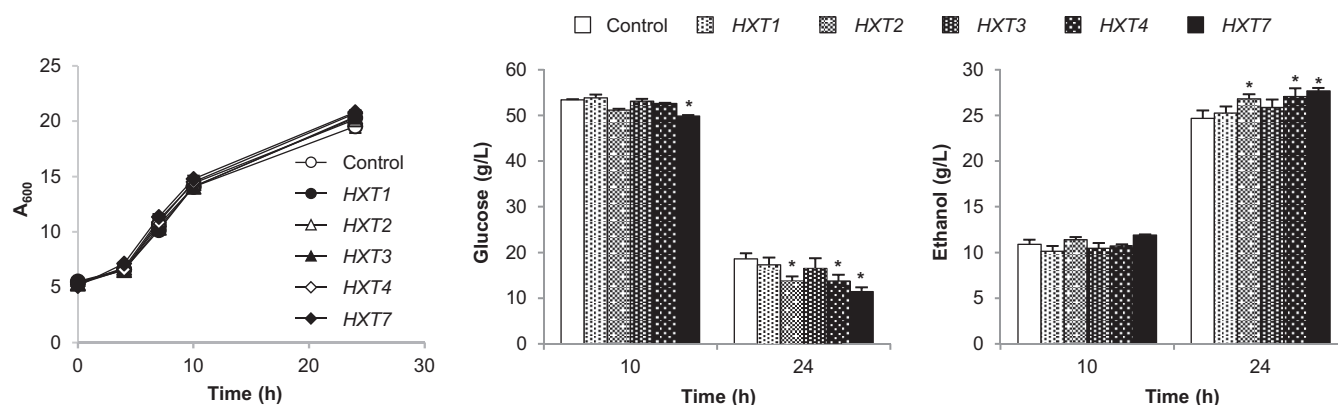
FIG 1 Increased glucose uptake and ethanol production rates by overexpression of *HXT* genes. CEN.PK2-1C cells overexpressing each *HXT* gene (*HXT1* to *HXT4* and *HXT7*) were grown in SC-Trp medium with 80 g/liter glucose, and glucose and ethanol levels in the medium were detected after 10 and 24 h of cultivation. Cells harboring empty pRS414GPD vector were used as a control. Mean values of the results of triplicate experiments are shown with error bars indicating the relative standard deviations. *, $P < 0.05$ (compared to the wild type).

into 50 ml YPD medium containing 4% glucose with or without 5 g/liter CaCO$_3$ in 250-ml flat-capped flasks and grown at 30°C under microaerobic conditions with shaking at 90 rpm.

Construction of plasmids. Plasmids used in this study and primers used for the construction of the plasmids are described in Table 1 and Table 2, respectively. *HXT1*, *HXT2*, *HXT3*, and *HXT4* ORFs were amplified by PCR using the primers shown in Table 2 and were inserted between the BamHI and ClaI sites of the pRS414GPD plasmid vector (33). The *HXT7* ORF was inserted between the BamHI and XhoI sites of pRS414GPD, and *GCR1* and *GCR2* ORFs were inserted between the XbaI and XhoI sites of pRS416GPD and pRS415GPD, respectively. The P$_{TDH3}$-*GCR1*-T$_{CYC1}$ fragment was amplified from pRS416GPD-*GCR1* by PCR using primers c_*GCR1* F and c_*GCR1* R and cloned into the KpnI site of pUC57-URA3 (29), generating pUC57-URA3-*GCR1*.

qRT-PCR analysis. Total RNA was isolated from yeast cells, and the relative amounts of specific mRNA were determined by quantitative reverse transcription-PCR (qRT-PCR). Briefly, 1 mg of the total RNA was subjected to reverse transcription in a 20-μl reaction mixture containing 200 U of myeloblastosis virus reverse transcriptase (M-biotech, South Korea) and 0.1 mg of oligo(dT) at 42°C for 1 h. Quantitative PCR was performed with a LightCycler 480 II instrument (Roche Diagnostics, Germany) using SYBR green PCR master mix (Roche Diagnostics, Germany) and gene-specific primers (Table 2) under the following conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s, and cooling to 40°C for 30 s. All experiments were performed in triplicate, and the data were normalized using the *ACT1* gene as a control.

Detection of metabolites. To quantify the concentrations of glucose, ethanol, and lactic acid, 600 μl to approximately 1 ml of culture supernatants was collected and filtered through a 0.22-μm-pore-size syringe filter. High-performance liquid chromatography (HPLC) analysis was performed in an UltiMate 3000 HPLC system (Thermo Fisher Scientific) equipped with a Bio-Rad Aminex HPX-87H column (300 by 7.8 mm, 5 μm pore size) at 60°C with 5 mM H$_2$SO$_4$ and a flow rate of 0.6 ml/min and in a refractive index (RI) detector.

RESULTS

Effects of overexpressing HXTs with different glucose affinities on the glucose uptake rate. In a previous study, it was shown that overexpression of *HXT1* and *HXT7*, encoding low- and high-affinity transporters, increases the glucose uptake rate with similar efficiencies (22). We reinvestigated the effects of HXT overexpression on the glucose uptake rate by individually overexpressing 5 *HXT* genes, *HXT1*, *HXT2*, *HXT3*, and *HXT4* and *HXT7*, encoding

glucose transporters with different affinities. The *HXT* genes were overexpressed under the control of a strong constitutive *TDH3* promoter from a centromere (CEN)/autonomously replicating sequence (ARS)-based low-copy-number plasmid vector. In SC-Trp minimal medium containing 80 g/liter glucose, CEN.PK2-1C cells expressing each *HXT* gene showed higher glucose uptake and ethanol production rates than did cells of a control strain harboring an empty plasmid vector (Fig. 1). The amounts of glucose consumed in each strain after 10 and 24 h of cultivation were correlated with the amounts of ethanol produced, reflecting the fact that ethanol production is the major carbon flux during the yeast fermentation. Overexpression of high-affinity glucose transporter Hxt7 showed the greatest effect, followed by moderate-affinity glucose transporters Hxt2 and Hxt4. Overexpression of low-affinity transporters Hxt1 and Hxt3 showed marginal effects on glucose uptake and ethanol production (Fig. 1).

Effects of deleting Mth1 and Std1, corepressors of *HXT* genes, on the glucose uptake rate. Mth1 and Std1 act as corepressors of the repressor Rgt1, whose target genes include *HXT1* to *HXT4* and *HXT7* (16, 19). Although Std1 and Mth1 are mainly functional in the absence of glucose, deletion of *STD1* and/or *MTH1* has been shown to derepress *HXT* genes simultaneously to different extents depending on glucose concentrations (18). Therefore, we deleted *STD1* and *MTH1* individually or in combination to examine the effects of multiple upregulation of *HXT* genes on glucose uptake.

In SC medium, glucose uptake and ethanol production rates were slightly higher in the *mth1*Δ and *std1*Δ strains than in the wild type (Fig. 2A). The effect of double deletion was very subtle compared with those of single deletions (Fig. 2A). However, in rich YPD medium, the *std1*Δ and *mth1*Δ *std1*Δ strains showed a significant increase in glucose uptake and ethanol production rates compared with the wild type (Fig. 2B). The *mth1*Δ *std1*Δ strain, showing the highest glucose uptake rate, also showed a growth rate higher than those of the other strains. To elucidate the HXTs responsible for the differences in glucose uptake rates, we measured mRNA levels of *HXT* genes in each strain after 6 h of cultivation. *HXT1* mRNA levels showed the most noticeable derepression in the *std1*Δ and *mth1*Δ *std1*Δ strains, suggesting that the increased expression of *HXT1* might be the factor mainly re-
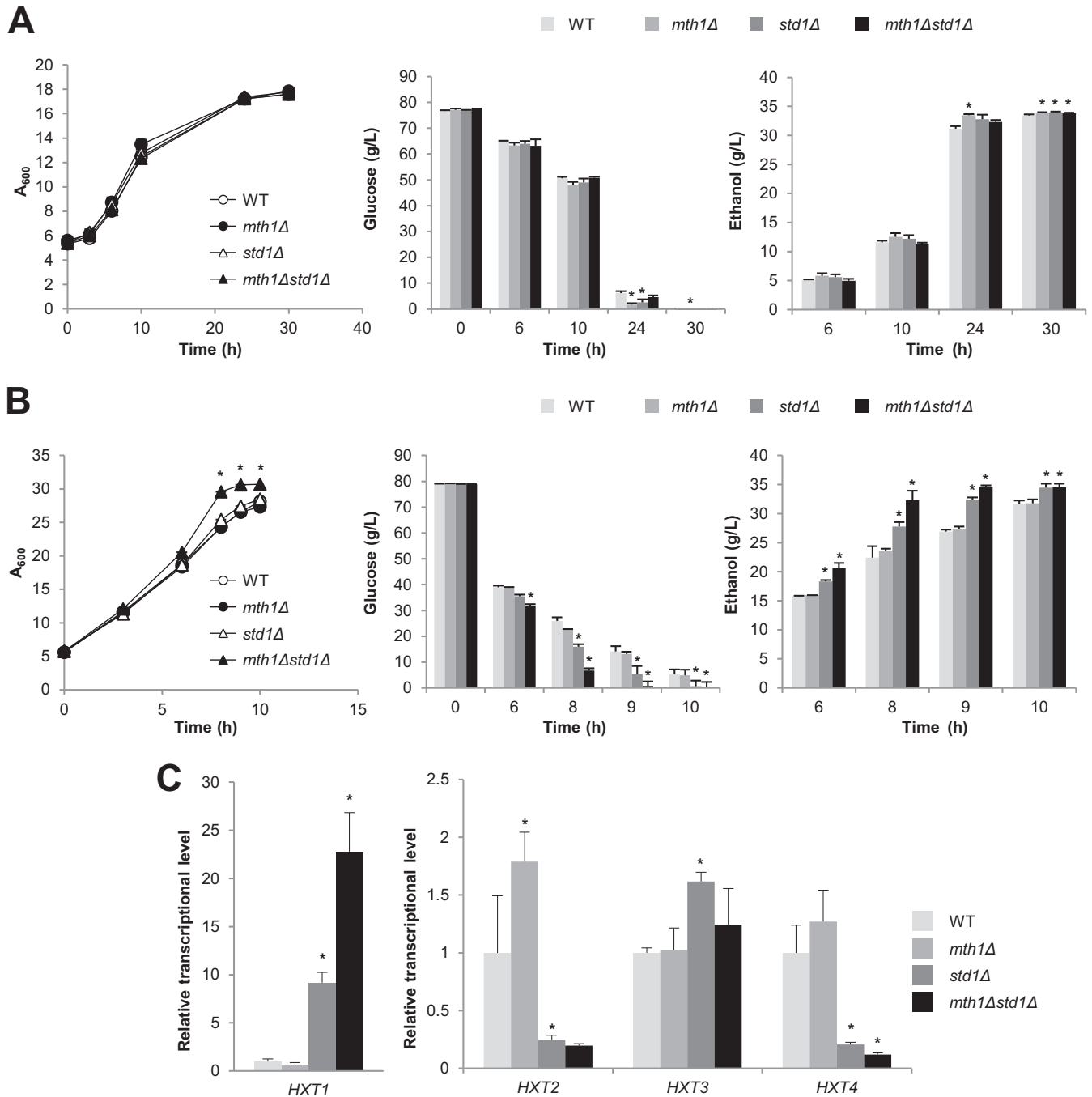
FIG 2 Effects of deleting corepressor genes *MTH1* and *STD1* on glucose uptake and ethanol production rates. Wild-type (WT; CEN.PK2-1C) and *mth1Δ*, *std1Δ*, and *mth1Δ std1Δ* *S. cerevisiae* cells were grown in SC (A) or YPD (B) medium with 80 g/liter glucose, and glucose and ethanol levels were detected during growth. (C) Expression levels of *HXT* genes. The indicated strains were grown in YPD (80 g/liter) medium for 6 h, and the mRNA expression levels of each *HXT* gene were measured by qRT-PCR and normalized to that of the *ACT1* gene. The mRNA levels relative to that of the wild-type control are indicated with standard deviations of the results of three independent experiments. *, $P < 0.05$ (compared to the wild type).

sponsible for the enhanced glucose uptake in these strains in YPD medium (Fig. 2C). *HXT3* expression also slightly increased in the *std1Δ* strain but not in the *mth1Δ std1Δ* strain (Fig. 2C). In contrast, the expression levels of *HXT2* and *HXT4* decreased in the *std1Δ* strain and the *mth1Δ std1Δ* strain (Fig. 2C).

Taking the results together, the effects of deleting *MTH1* and/or *STD1* on the expression of *HXT* genes and glucose uptake

appear to be variable, depending on culture conditions, which might be due to the condition-dependent expression levels of Mth1 and Std1 as well as the complex regulatory networks of the Mth1/Std1-Rgt1 transcriptional regulators, which regulate not only *HXT* genes but also other target genes.

Improvement of the glucose uptake rate by overexpressing GCR1. In previous studies, overexpression of genes encoding gly-

colytic enzymes was not effective in significantly improving gly-colytic flux (23–27). Therefore, we tested whether overexpression of transcription factor Gcr1, which activates not only glycolytic genes but also other glucose-responsive target genes such as ribosomal protein (RP) genes, could improve the glucose uptake rate through facilitating glucose utilization and cell growth (34). Gcr1 binds to the CT (CTTCC) box in the promoters of glycolytic genes, and Gcr2 interacts with Gcr1 for the transcriptional activation. However, Gcr2 is dispensable for the activation of RP gene transcription by Gcr1 (34). CEN.PK2-1C cells overexpressing *GCR1* showed increased glucose uptake and ethanol production rates (Fig. 3A). In contrast, overexpression of *GCR2* decreased the glucose uptake rate somewhat, and overexpression of both *GCR1* and *GCR2* resulted in significant defects in growth and glucose uptake (Fig. 3A).

To elucidate the effects of *GCR1* overexpression, transcription levels of its potential target genes were investigated after 12 h of cultivation. In contrast to our initial expectation, transcription levels of glycolytic genes (*ENO1*, *GPM1*, and *PGK1*) were not elevated in cells overexpressing *GCR1* (Fig. 3B). On the other hand, overexpression of *GCR1* led to an increase in expression levels of RP genes (*RPL11A*, *RPS18B*, and *RPL30*), *HXT1*, and *ADH1* (encoding alcohol dehydrogenase) (Fig. 3B). Expression levels of *HXT2*, *HXT3*, and *HXT4* were not significantly changed by *GCR1* overexpression (data not shown). Therefore, *GCR1* overexpression might increase the glucose uptake rate mainly by enhancing ribosome biogenesis, which is tightly linked to cell growth, rather than by elevating the expression levels of glycolytic genes.

Overexpression of *GCR1* was more efficient in enhancing the glucose uptake rate than overexpression of *HXT7* (Fig. 3C). Slight additive effects were observed when the *HXT7* gene was overexpressed together with *GCR1* (Fig. 3C). Although the control cells harboring empty vector(s) showed glucose uptake rates in SC-Trp medium (Fig. 1) and SC-Trp-Ura medium (Fig. 3C) that were lower than that in SC-Leu-Ura medium (Fig. 3A), the effects of overexpression of *HXT7* and *GCR1* on the glucose uptake rate were reproducibly observed independently of the types of medium.

Improvement of lactic acid production by overexpressing *GCR1*. Since overexpression of *GCR1* was proven to be effective in increasing glucose uptake and ethanol production rates in wild-type *S. cerevisiae*, we next applied *GCR1* overexpression to lactic acid production in engineered strains expressing lactate dehydrogenase. We used two lactic acid-producing strains, SP2006 and SP1130 (29). For high-yield lactic acid production, the pathways for glycerol and ethanol formation had been attenuated in SP2006 by deleting *GPD1* (encoding glycerol-3-phosphate dehydrogenase), *PDC1* (encoding pyruvate decarboxylase), and *ADH1* (encoding alcohol dehydrogenase). In SP2006, insufficient production of acetyl-coenzyme A (CoA), a key precursor for the biosynthesis of various cellular components, could be one of the growth-limiting factors. Therefore, SP1130, an improved derivative of the construction of SP2006, was generated by increasing the cellular supply of acetyl-CoA via the introduction of bacterial genes for acetylating acetaldehyde dehydrogenase (A-ALD) enzymes MphF and EutE while deleting *PDC6* encoding aldehyde dehydrogenase (29). In the present work, *GCR1* was overexpressed in SP2006 and SP1130 by integrating the gene into the genome under the control of the *TDH3* promoter, generating SP2018 and SP1141, respectively.

Interestingly, overexpression of *GCR1* exhibited different effects depending on the lactic acid production ability of each strain. Note that the amounts of glucose uptake in the lactic acid-producing strains were limited to only 27.4 g/liter and 30.2 g/liter glucose, respectively, when SP2006 and SP1130 strains were grown in YPD medium containing 40 g/liter glucose (Fig. 4A and B). The volumetric productivity of lactic acid decreased over time in both SP2006 and SP1130, but SP1130 showed about 20% higher productivity than SP2006 at up to 28 h of cultivation (Fig. 4A and B). SP2006 produced 16.2 g/liter lactic acid after 28 h, whereas SP1130 produced 19.5 g/liter. After further cultivation to 72 h, SP2006 and SP1130 produced 19.8 g/liter and 21.5 g/liter lactic acid, respectively. Overexpression of *GCR1* in SP2006 (strain SP2018) led to a slight increase in the glucose uptake level and final cell density (Fig. 4A). However, the increased amount of glucose transported into the SP2018 cells was not efficiently converted to lactic acid, with slightly decreased lactic acid production levels shown instead. On the other hand, *GCR1* overexpression in the SP1130 background (strain SP1141) resulted in a significant increase in glucose consumption and lactic acid production levels (Fig. 4B). By overexpression of *GCR1*, glucose consumption increased from 30.2 g/liter to 38.4 g/liter and lactic acid production increased from 21.5 g/liter to 28.9 g/liter compared to the results seen with strain SP1130. The yield also increased from 0.71 g/g glucose to 0.75 g/g glucose.

Next, we investigated whether the growth-promoting effect of *GCR1* overexpression could also improve lactic acid production when the growth inhibitory acid stress was relieved by neutralization. When SP1130 cells were grown in YPD medium containing 40 g/liter glucose and 5 g/liter $CaCO_3$ as a neutralizing reagent, the cells were found to have consumed all of the glucose in the medium after 40 h, producing 27.6 g/liter lactic acid. Under these fermentation conditions, SP1141 showed glucose uptake rates and lactic acid production levels similar to those shown by SP1130, suggesting that *GCR1* overexpression might provide a selective advantage when cell growth is impaired.

Taking the data together, *GCR1* overexpression might contribute to improving cell growth by facilitating glucose uptake and protein synthesis. However, the positive correlation between the growth-promoting effect and the production of a target chemical might be prominent only in the presence of efficient and strong metabolic flux toward the target chemical and when growth inhibition is one of the limiting factors for the production of the target chemical.

DISCUSSION

Glucose uptake is the first rate-limiting step to control cellular metabolism, depending on extracellular glucose availability and intracellular metabolic capacity. Very sophisticated regulatory mechanisms for glucose uptake have been evolved to ensure cell survival in natural environments with high fluctuation of glucose availability (6). However, engineering the natural regulatory networks to allow a higher glucose uptake rate might be beneficial for the production of various fuels and chemicals if the engineered strain has an efficient metabolic flux toward a target chemical.

As an effort to increase the glucose uptake rate, we tested various genetic modifications related to glucose uptake and cell growth. The most direct strategy was overexpressing HXTs with various affinities to glucose. Among the HXTs tested (Hxt1, Hxt2, Hxt3, Hxt4, and Hxt7), high-affinity transporter Hxt7 was most
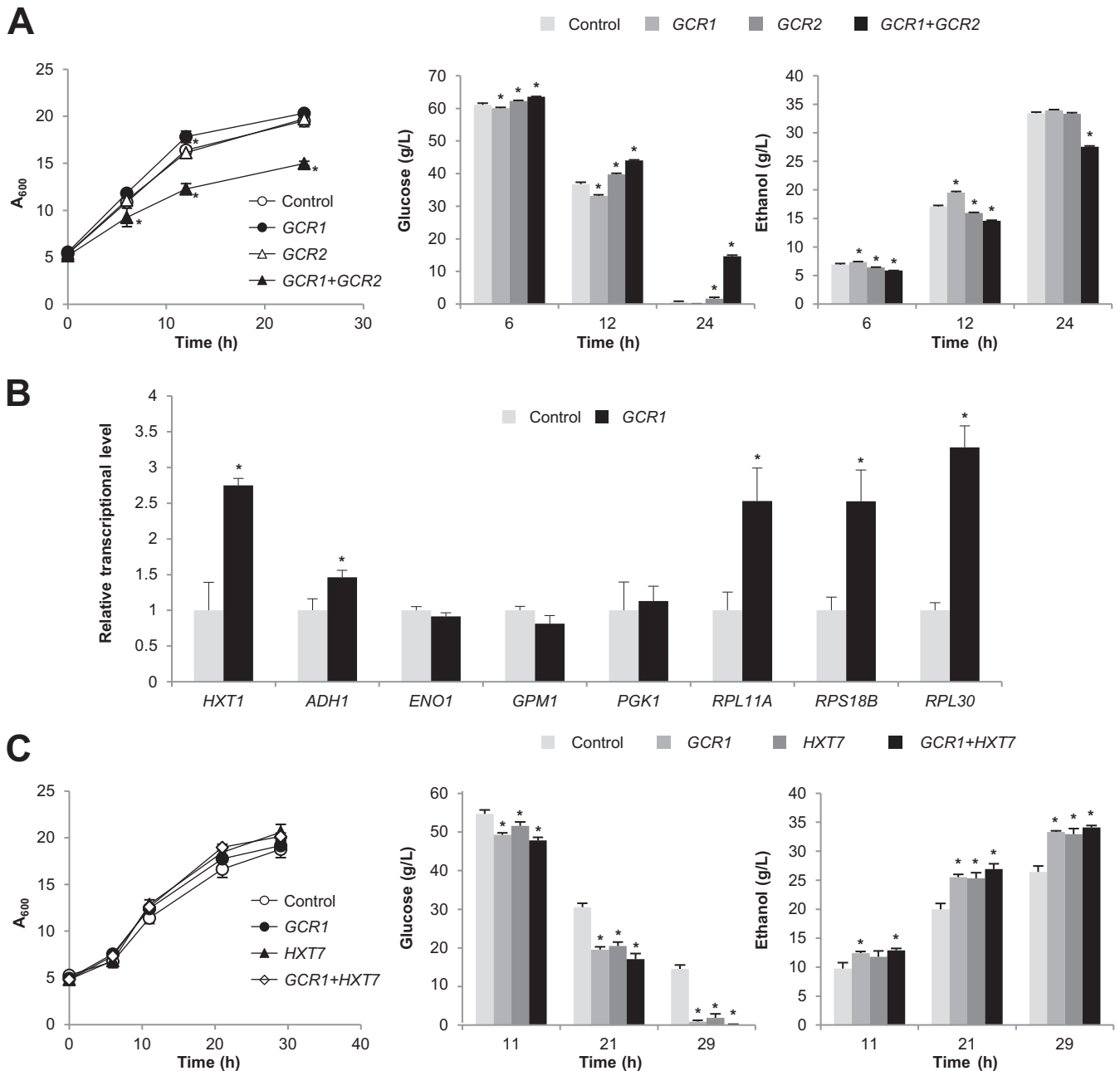
FIG 3 Improvement of glucose uptake rate by overexpressing *GCR1*. (A) Effects of overexpressing *GCR1* and/or *GCR2* on the glucose uptake rate. CEN.PK2-1C cells overexpressing *GCR1* and *GCR2* alone or in combination were grown in SC-Leu-Ura medium with 80 g/liter glucose and monitored for glucose uptake and ethanol production. Cells expressing empty vectors pRS415GPD and pRS416GPD were used as a control. (B) Effects of overexpression of the *GCR1* gene on the transcription levels of its target genes. CEN.PK2-1C cells overexpressing *GCR1* or harboring pRS416GPD (control) were grown in SC-Ura medium with 80 g/liter glucose for 12 h, and mRNA expression levels of Gcr1 target genes were measured by qRT-PCR and normalized to the *ACT1* level. The mRNA levels relative to those of the control are indicated with standard deviations of the results of three independent experiments. *, $P < 0.05$ (compared to control). (C) Effects of overexpressing *GCR1* alone or in combination with *HXT7*. Cells overexpressing the indicated genes were grown in SC-Trp-Ura medium with 80 g/liter glucose and monitored for glucose uptake and ethanol production. Cells expressing empty vectors (pRS414GPD and pRS416GPD) were used as a control. Mean values of triplicate experiments are shown with error bars indicating standard deviations. *, $P < 0.05$ (compared to control).

effective in increasing the glucose uptake rate, followed by moderate-affinity transporters Hxt2 and Hxt4. The effects of eliminating Mth1 and Std1 corepressors of *HXT* genes were more complicated due to the complex nature of the regulatory networks and the multiple targets regulated by Rgt1 (19). Mth1 and Std1 are homologous proteins acting as corepressors of Rgt1, but their cel-

lular functions are distinguished mainly on the basis of their differential expression levels depending on glucose concentrations (35). Although expression levels of Std1 are not much affected by glucose concentrations, Mth1, whose expression is repressed by the Snf1-Mig1 glucose repression pathway, is abundant only under glucose starvation conditions (17, 35, 36). The effects of dele-
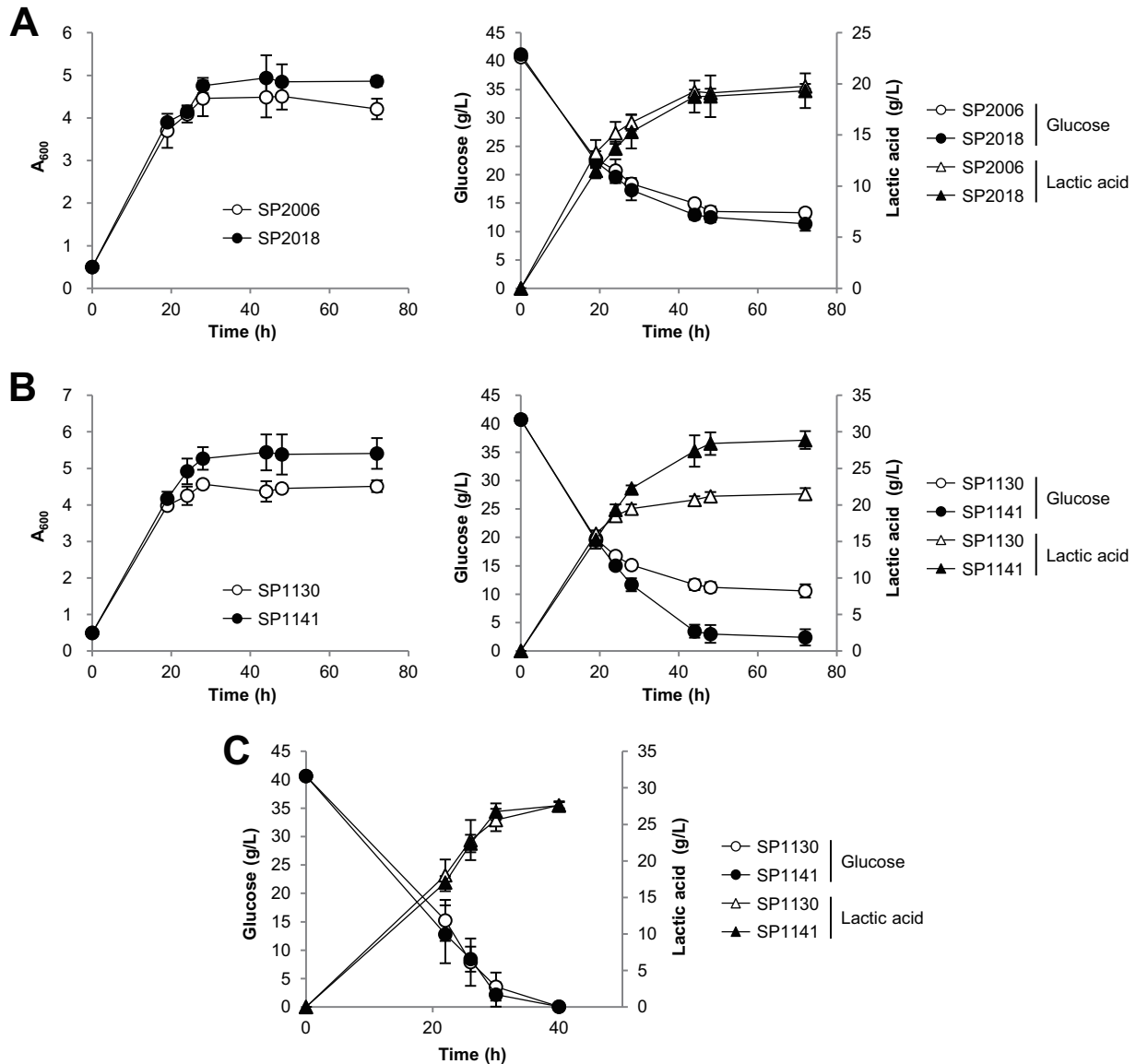
FIG 4 Improvement of lactic acid production by overexpressing *GCR1*. (A) Effects of *GCR1* overexpression in strain SP2006 background. SP2006 and SP2018 (SP2006 overexpressing *GCR1*) strains were grown in YPD medium containing 40 g/liter glucose under microaerobic conditions. (B and C) Effects of *GCR1* overexpression in strain SP1130 background. Glucose uptake and lactic acid production were measured in SP1130 and SP1141 (SP1130 overexpressing *GCR1*) strains during growth in YPD medium containing 40 g/liter glucose without $CaCO_3$ (B) or with 5 g/liter $CaCO_3$ (C) under microaerobic conditions. Mean values of the results of triplicate experiments are shown with error bars indicating standard deviations.

tion of *MTH1* and/or *STD1* on glucose uptake were more prominent in YPD medium than in SC medium. In SC medium, deletion of *MTH1* was most effective in increasing the glucose uptake rate, but in YPD medium, the *std1Δ* and *std1Δ mth1Δ* strains, but not the *mth1Δ* strain, showed an increased glucose uptake rate. Such culture condition-dependent effects of *MTH1* and/or *STD1* deletions on glucose uptake might reflect different levels of expression of Mth1 and Std1 as well as their slightly different regulatory mechanisms, which lead to different levels of contributions of each corepressor to the regulation of Rgt1 target genes under a given set of conditions. Different nutritional compositions and pH conditions of YPD and SC media and the faster pH drop in SC medium than in YPD medium might be responsible for the differential roles of Mth1 and Std1. In YPD medium,

derepression of *HXT1* seems to have been mainly responsible for the increase in glucose uptake rate in the *std1Δ* and *std1Δ mth1Δ* strains under our experimental conditions, although we cannot rule out the contributions of other Rgt1 target genes. Unexpectedly, the *std1Δ* and *std1Δ mth1Δ* strains exhibited reduced expression levels of *HXT2* and *HXT4*, which might have been due to indirect effects of derepressing other Rgt1 target genes such as *MIG2*, which encodes a repressor for *HXT2* and *HXT4* (10, 35).

As another strategy to increase the glucose uptake rate, we overexpressed *GCR1*. Gcr1 plays an important role in glucose metabolism by activating glycolytic genes, *HXT* genes, and *ADH1* (37). Furthermore, Gcr1 also promotes cell growth by activating the expression of translational components such as RP genes (34). Gcr2, a Gcr1-binding coactivator, is essential only for the expres-

sion of CT box-containing glycolytic genes, but not for RP genes, which do not have a CT box (34). Although overexpression of *GCR1* was effective in increasing glucose uptake and ethanol production rates, overexpression of *GCR2* alone or in combination with *GCR1* showed a negative effect on glucose uptake. Overexpression of *GCR1* led to increased expression levels of *HXT1*, *ADH1*, and RP genes but not glycolytic genes. These results might be in part related to the fact that transcriptional activation of glycolytic genes requires both Gcr1 and Gcr2 but Gcr1 alone can activate RP gene transcription. Therefore, the increase in the glucose uptake rate mediated by *GCR* overexpression might be caused by improving cell growth through promoting translational ability and by increased expression of *HXT1* rather than by increasing glycolytic flux through activating the expression of glycolytic genes.

Overexpression of *GCR1* was successfully applied to increase lactic acid production in engineered yeast strain SP1141. Inside lactic acid-producing cells, lactic acid molecules dissociate into acid anions and protons, inducing multiple stress conditions via cytosolic acidification and modifications of cellular components (38, 39). Furthermore, exporting the protons and acid anions through H^+-ATPase and efflux pumps is a highly energy-consuming process (40). Because of such toxic effects of lactic acid, cell growth and glucose uptake halt when lactic acid production reaches a certain level. SP1130, the parental strain of SP1141, consumed only 30.2 g/liter glucose even in the presence of 40.0 g/liter glucose in the medium, producing 21.5 g/liter lactic acid. On the other hand, strain SP1141, overexpressing *GCR1*, showed increased glucose uptake up to 38.4 g/liter, and the imported glucose was successfully converted to lactic acid, reaching 28.9 g/liter. Therefore, the growth-promoting effect of *GCR1* overexpression might increase the threshold level of the growth-inhibitory effects of lactic acid, leading to higher titers, yield, and efficiency of lactic acid production. However, *GCR1* overexpression was not effective when applied to another lactic acid-producing strain, SP2006, which has a lower acetyl-CoA level and weaker lactic acid-producing capability than SP1130. Therefore, in SP2018, with inefficient metabolic flux to lactic acid, the increased amount of imported glucose might be mainly used for biomass synthesis rather than for lactic acid production.

Although *GCR1* overexpression was effective in improving cell growth, it failed to exert a positive role for lactic acid production when the growth-inhibitory effect of the acid stress was alleviated by neutralizing the medium during the fermentation. Therefore, Gcr1 overexpression could be applied to improve lactic acid production during acidic fermentation. Acidic fermentation is preferred for lactic acid production to reduce the cost of neutralization during fermentation and recovery of lactic acid from the resulting lactate salt (33).

Taking the data together, increasing glucose uptake and cell growth rates might be a very useful strategy to improve the production of target metabolites if efficient metabolic pathways were to be provided to synthesize the desired molecules. Although overexpression of HXTs might affect only the glucose uptake rate, overexpression of *GCR1* appears to exert an additional growth-facilitating effect by activating the transcription of RP genes. Although promoting cell growth and biomass synthesis might seem unfavorable in view of the goal of increasing the yield of a target chemical, improving cell growth could act in a positive way if impaired growth is a limiting factor for the production of a target chemical. Therefore, *GCR1* overexpression might be a promising strategy for many other metabolic engineering applications where growth inhibition is one of the major bottlenecks impeding improved production.

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