GSF2 deletion increases lactic acid production by alleviating glucose repression in Saccharomyces cerevisiae

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Improving lactic acid (LA) tolerance is important for cost-effective microbial production of LA under acidic fermentation conditions. Previously, we generated LA-tolerant D-LA-producing S. cerevisiae strain JHY5310 by laboratory adaptive evolution of JHY5210. In this study, we performed whole genome sequencing of JHY5310, identifying four loss-of-function mutations in GSF2, SYN8, STM1, and SIF2 genes, which are responsible for the LA tolerance of JHY5310. Among the mutations, a nonsense mutation in GSF2 was identified as the major contributor to the improved LA tolerance and LA production in JHY5310. Deletion of GSF2 in the parental strain JHY5210 significantly improved glucose uptake and D-LA production levels, while derepressing glucose-repressed genes including genes involved in the respiratory pathway. Therefore, more efficient generation of ATP and NAD+ via respiration might rescue the growth defects of the LA-producing strain, where ATP depletion through extensive export of lactate and proton is one of major reasons for the impaired growth. Accordingly, alleviation of glucose repression by deleting MIG1 or HXK2 in JHY5210 also improved D-LA production. GSF2 deletion could be applied to various bioprocesses where increasing biomass yield or respiratory flux is desirable.

Microbial production of lactic acid (LA) has received a great attention for the production of poly lactic acid (PLA), a biodegradable polymer1–3. Lactic acid bacteria naturally produce LA, but neutralizing reagent such as CaCO3 should be added during fermentation due to their acid sensitivity. Such a neutralizing fermentation process requires recovery of LA from the resulting calcium salt of lactate by treating sulfuric acid, producing gypsum as an undesirable byproduct4–6. Therefore, Saccharomyces cerevisiae having higher acid tolerance than lactic acid bacteria is considered a promising host for LA production5–9. However, even in S. cerevisiae, growth inhibition caused by LA accumulation is the major limiting factor preventing high-titer production of LA7,8. Under acidic conditions, undissociated LA molecules in the medium diffuse through the plasma membrane and dissociate into the acid anions and protons in the cytosol, where the pH is neutral. This leads to growth-inhibitory stress conditions including cytosolic acidification, modifications of cellular components, and energy depletion from the excessive use of ATP to export protons and lactate ions5,6,8,10,11. Therefore, to produce LA without neutralization during fermentation, it is critical to improve LA tolerance.

Cellular responses to weak acids such as acetic acid, lactic acid, benzoic acid, and sorbic acid are variable depending on the chemical properties of weak acids12,13. Transcriptome analyses in S. cerevisiae have revealed that Aft1 transcription factor plays an important role in induction of genes involved in iron homeostasis in the presence of lactate anion, which might reflect iron chelating activity of lactate10,14. On the other hand, Haa1 transcriptional activator is mainly responsible for cellular response to undissociated lactic acid10,15. Accordingly, we previously showed that overexpression of Haa1 could improve LA tolerance and LA production under acidic fermentation conditions9,16. Genome-wide screening of nonessential deletion strain collection or RNAi-mediated knockdown library discovered several genes whose deletion or knockdown could improve LA tolerance9,14,17. The identified genes cover a wide range of biological functions such as cell wall components, histone acetyltransferase

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complex, and a ribosome-associated chaperone, implying the complexity of cellular defense mechanisms against LA stress. LA tolerance can be improved by logical genetic modifications based on the stress-tolerance mechanisms. However, considering the fact that LA tolerance mechanisms are not fully understood and involve complex networks of multiple genes, adaptive laboratory evolution is another efficient strategy to obtain tolerant strains. This can be a powerful tool in combination with whole genome sequencing analysis and reverse metabolic engineering for the identification of modified genes and pathways, which are difficult to predict rationally.

In this study, we identified genes involved in LA tolerance from genome sequencing of LA-tolerant strain JHY5310, generated by adaptive evolution in our previous study. We demonstrated that alleviating glucose repression by \textit{GSF2} deletion can significantly improve LA tolerance and LA production possibly by eliciting more efficient ATP synthesis via respiratory pathway.

**Results**

**Whole genome sequencing analysis of LA-tolerant strain JHY5310.** Previously, we generated D-LA-producing \textit{S. cerevisiae} strain JHY5210 (\textit{ddl1 Δjen1 Δadh1 Δgpd1 Δgpd2 Δpdc1 Δ:Leuconostoc mesenteroides ATCC 8293} and by deleting \textit{DLI1} encoding D-lactate dehydrogenase, \textit{JEN1} encoding monocarboxylate transporter, and major competing pathways producing ethanol and glycerol). In addition, from adaptive laboratory evolution of the strain JHY5210, we isolated strain JHY5310 with improved LA tolerance (Fig. 1). JHY5310 also showed improved growth even in the absence of LA in the medium (Fig. 1).

To identify genes involved in LA tolerance in JHY5310, whole genome sequencing of JHY3310 and its parental strain JHY5210 was carried out. In comparison with JHY5210, mutations in \textit{GSF2}, \textit{SYN8}, \textit{STM1}, and \textit{SIF2} genes were identified in JHY5310 (Table 1). \textit{GSF2} in the evolved strain has a novel stop codon by point mutation at position 4, immediately after start codon. A nonsense mutation was also found in \textit{SYN8}, which changes the codon for Glu121 to stop codon. \textit{STM1} gene has a 17-bp internal deletion from position 417 to 433, resulting in a frameshift mutation after Asp140. A missense mutation was found in \textit{SIF2}, resulting in Met to Ile substitution at 66 amino acid residue.

\textit{Gsf2}, an integral membrane protein localized in the endoplasmic reticulum (ER), is known to be involved in maturation and secretion of certain type of hexose transporters such as Hxt1 and Gal2 to the plasma membrane. \textit{Syn8} is an endosomal SNARE protein, and deletion of \textit{SYN8} was previously reported to increase LA tolerance. \textit{Stm1}, a protein required for facilitating translation under nutrient stress condition, is known to be

**Figure 1. Identification of genes responsible for LA tolerance of JHY5310.** Unevolved parental strain JHY5210, deletion strains derived from JHY5210, and evolved strain JHY5310 were grown in YPD medium and then OD$_{600}$ of 1 cells were serially diluted and spotted onto YPD solid medium with or without 1.5% LA.

| Gene  | Type of mutation | Nucleotide change | Amino acid change |
|-------|------------------|------------------|------------------|
| \textit{GSF2} | Nonsense (GAG $\rightarrow$ TAG) | 4G $\rightarrow$ T | Glu2 $\rightarrow$ Stop |
| \textit{SYN8} | Nonsense (GAG $\rightarrow$ TAG) | 361G $\rightarrow$ T | Glu121 $\rightarrow$ Stop |
| \textit{STM1} | Frameshift | Δ417–433 | Frameshift after Asp140 |
| \textit{SIF2} | Missense (ATG $\rightarrow$ ATT) | 198G $\rightarrow$ T | Met66 $\rightarrow$ Ile |

**Table 1. Mutations in the JHY5310 genome.**
associated with apoptosis and telomere biosynthesis. Sif2 encodes a component of Set3C histone deacetylase complex. Any gene duplication or insertion was not found in the evolved strain JHY5310.

**Effects of mutated genes on LA tolerance and LA production.** The identified nonsense mutations (GSF2 and SYN8) and a frameshift mutation (STM1) might be loss-of-function mutations. Therefore, we first investigated the effects of deleting the mutated genes on LA tolerance. GSF2, SYN8, STM1, and SIF2 genes were deleted in the parental strain JHY5210, and their growth was compared on YPD solid medium in the presence or absence of 1.5% LA (Fig. 1). All deletion strains showed enhanced LA tolerance compared to JHY5210, although to a less extent than the evolved strain JHY5310. Among the four genes, deletion of GSF2 was most effective in enhancing LA tolerance. These results suggest that the identified four mutations might be loss-of-function mutations that all contribute to the LA tolerance of JHY5310. On the other hand, the deletion strains showed different growth rates on control YPD medium without LA. Compared to JHY5210, JHY5212 (gsf2Δ) and JHY5215 (sif2Δ) showed improved cell growth, whereas JHY5214 (stm1Δ) showed a slight growth defect under normal conditions (Fig. 1). Therefore, deletion of GSF2 and SIF2 might improve cellular fitness of JHY5210, whereas the deletion effects of SYN8 and STM1 might be more specific to cellular defense against LA stress.

Next, we examined the effect of each gene on LA production. Although all deletion strains showed enhanced LA tolerance, only JHY5212 showed significantly higher glucose consumption and LA production levels compared to JHY5210 (Fig. 2a), which is consistent with the biggest effect of GSF2 deletion on LA tolerance (Fig. 1). We also tested the effects of overexpressing the identified genes. None of these genes showed any significant improvement of glucose consumption and D-LA production when overexpressed, supporting the idea that LA tolerance is the result of inactivation of these genes in JHY5310 (Fig. 2b).

**GSF2 deletion improved LA production by alleviating glucose repression.** Since strain JHY5212 showed the best performance in LA production, we compared this strain with the evolved strain JHY5310. When the unevolved strain JHY5210 was cultured in YPD medium containing 50 g/L glucose, only 28.9 g/L glucose was consumed, producing 16.9 g/L D-LA with a yield of 0.58 g/g glucose (Fig. 3). Medium pH dropped from 6.6 to 3.2 during the fermentation (Supplementary Figure S1), supporting the idea that acidification of the medium might be a critical growth inhibitory factor. On the other hand, the evolved strain JHY5310 consumed 49.3 g/L of glucose, producing 36.8 g/L D-LA with a yield of 0.75 g/g glucose (Fig. 3). JHY5212 showed slightly lower glucose consumption (45.8 g/L) and D-LA production (33.2 g/L) levels than did JHY5310 (Fig. 3). Taken together, these results suggest that the nonsense mutation of GSF2 is the major contributor to the enhanced LA tolerance and LA production in the evolved strain JHY5310, with minor contributions of 3 other mutations identified in the genome.

Gsf2, an ER membrane protein, is known to be involved in the transportation of a subset of hexose transporters such as Hxt1 to the plasma membrane. Therefore, GSF2 deletion leads to a decrease in functional localization of Hxt1 in the plasma membrane, resulting in reduced glucose uptake rate, which in turn can alleviate glucose repression. In S. cerevisiae, glucose repression is the major regulatory mechanism of deriving high glucose flux toward ethanol fermentation while inhibiting respiratory growth even under aerobic conditions. Therefore, considering the fact that ATP depletion caused by extensive use of H+–ATPase and efflux pumps to export protons and lactate anions is one of the major reasons for growth inhibition upon accumulation of LA, more efficient ATP synthesis through respiration might be responsible for the improved growth of JHY5212 during LA production. In addition, since ethanol and glycerol production pathways were largely blocked in JHY5210, NAD+ regeneration via heterologous lactate dehydrogenase might not be sufficient for cell growth, and an increase in respiration might rescue the defect of NAD+ regeneration. In agreement with this idea, JHY5212 showed higher mRNA levels of COX6, NDH1, and SDH1 genes involved in the mitochondrial respiratory chain and TCA cycle than did JHY5210 (Fig. 4b–d). SUC2, another glucose-repressed gene, was also highly expressed in JHY5212 even in the presence of high level of glucose in the medium (Fig. 4e), supporting the proposed effect of GSF2 deletion on glucose derepression. In addition, expression of HXT1, which is also regulated by glucose repression, was slightly increased by GSF2 deletion (Fig. 4f), suggesting that the effect of GSF2 deletion on glucose derepression is not due to a reduced transcription level of HXT1. In contrast, in JHY5210, glucose-repressed genes were not derepressed until 47 h, suggesting that ATP synthesis via respiration might not be sufficient to circumvent ATP depletion.

**Glucose derepression by deleting MIG1 or HXK2 improved LA production.** Based on the hypothesis that GSF2 deletion in JHY5210 might increase LA production by relieving glucose repression, we tested whether other genetic modifications known to relieve glucose repression could also increase LA production. Mig1 is a well-known transcriptional repressor of the glucose-repressed genes. Hxk2 is a major cytosolic hexokinase involved in phosphorylation of glucose, but is also involved in transcriptional repression of glucose-repressed genes in the nucleus by interacting with Mig1. Both Mig1 and Hxk2 are negatively regulated by Snf1 kinase, an AMP-activated Ser/Thr kinase playing a central role in glucose derepression. Deletion of either MIG1 or HXK2 is known to alleviate glucose repression. Therefore, we deleted MIG1 or HXK2 in JHY5210, and tested for LA production capability. In agreement with our hypothesis, deletion of MIG1 or HXK2 led to increased glucose uptake and LA production levels compared to JHS210 (Fig. 5). However, the positive effect of MIG1 or HXK2 deletion on LA production was weaker than that of GSF2 deletion. Taken together, increasing glucose flux to respiratory pathway by alleviating glucose repression might be beneficial to improve LA production in JHY5210.

**GSF2 deletion in wild-type strain showed metabolic phenotypes of increased respiration.** Next, we investigated the effects of GSF2 deletion on cell growth of wild-type strain. GSF2 deletion in wild-type
strain CEN.PK2-1C (strain JHY5101) led to a slight, but significant ($P < 0.05$) decrease in specific growth rate ($\mu_{JHY5101} = 0.462 \pm 0.004 \text{ h}^{-1}$) compared to wild type ($\mu_{CEN.PK2-1C} = 0.485 \pm 0.010 \text{ h}^{-1}$), but increased final cell density (Fig. 6a), consistent with the fact that increasing respiratory capacity increases the biomass yield. In *S. cerevisiae*, specific glucose consumption rate shows a positive correlation with specific ethanol production rate (fermentation capability). During the exponential growth phase, *GSF2* deletion led to a 26% decrease in specific glucose consumption rate ($q_{\text{glucose}} = 5.31 \pm 0.07 \text{ mmol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ of dry biomass) and a 24% decrease in specific ethanol production rate ($q_{\text{ethanol}} = 10.01 \pm 0.11 \text{ mmol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ of dry biomass) compared to the parental strain CEN.PK2-1C ($q_{\text{glucose}} = 7.22 \pm 0.51 \text{ mmol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ of dry biomass and $q_{\text{ethanol}} = 13.11 \pm 0.72 \text{ mmol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ of dry biomass) (Fig. 6b). These results further support the hypothesis that the reduced specific glucose uptake rate in *GSF2* deletion strain increases glucose flux to respiration by relieving glucose repression.

**Figure 2.** Effects of gene deletion or overexpression on D-LA production and glucose consumption levels. (a) Indicated strains were cultured in YPD medium containing 50 g/L glucose. Cell growth and glucose and D-LA levels in the medium were detected. (b) JHY5210 cells harboring the indicated plasmid were grown in SC-Ura medium containing 50 g/L glucose. Cells harboring p416GPD plasmid were used as a control. Error bars indicate standard deviations of three independent experiments.
GSF2 deletion restores growth defects caused by insufficient NAD\(^+\) regeneration. We also tested whether GSF2 deletion in wild-type strain can increase LA tolerance. In contrast to the result observed in the LA-producing strain JHY5210, GSF2 deletion in wild type CEN.PK2-1C reduced LA tolerance on medium containing 2.5% LA (Fig. 6c). LA sensitivity was also observed in MIG1 and HXK2 deletion strains, implying that LA sensitivity might be a common phenotype of the glucose derepressed cells with increased respiration capacity (Fig. 6c). These results suggest that GSF2 deletion might increase LA tolerance in JHY5210 by rescuing its growth defects. Note that JHY5210 is much more sensitive to LA than CEN.PK2-1C. JHY5210 barely survived on YPD medium containing 1.5% LA (Fig. 1), whereas CEN.PK2-1C grew normally on the same medium (Fig. 6d).

In addition to the endogenously produced LA, insufficient NAD\(^+\) regeneration caused by blocking ethanol production pathway (\(adh1\Delta pdc1\Delta\)) and glycerol production pathway (\(gpd1\Delta gpd2\Delta\)) might contribute to the growth defects of JHY5210. Therefore, we investigated whether the growth defects of strains with impaired NAD\(^+\) regeneration, but without LA production, can be rescued by GSF2 deletion. JHY602 (\(adh1-5\Delta\)) lacking five genes encoding alcohol dehydrogenase (ADH) and JHY604 (\(adh1\Delta gpd1\Delta gpd2\Delta\)) having deletions of major ADH gene (\(ADH1\)) and genes involved in glycerol production (\(GPD1\) and \(GPD2\)) have growth defects due to the insufficient NAD\(^+\) regeneration. The growth defects of these strains on YPD medium were restored by GSF2 deletion (Fig. 6d). In particular, the growth rescuing effect of GSF2 deletion became more prominent in the presence LA in the medium (Fig. 6d), which might imply that respiration-dependent increase in ATP synthesis might be more critical for the survival of JHY602 and JHY604 under the conditions requiring higher ATP to circumvent LA toxicity.

Taken together, the effects of GSF2 deletion on LA tolerance might be dependent on the cellular metabolic status. For the cells having growth defects due to the impaired NAD\(^+\) regeneration or insufficient ATP synthesis, GSF2 deletion might contribute to improve LA tolerance.

Discussion
Increasing LA tolerance is one of the important issues to improve LA production under acidic fermentation conditions\(^6,8,9\). In our previous study, we enhanced LA tolerance of a D-LA-producing \(S.\ cerevisiae\) strain JHY5210...
by using adaptive laboratory evolution. In this study, we carried out whole genome sequencing analysis of the evolved strain JHY5310, followed by functional studies to identify mutated genes responsible for LA tolerance. We identified loss-of-function mutations in GSF2, SYN8, STM1, and SIF2 genes, which contribute to the LA tolerance of JHY5310. Among the four genes, deletion of GSF2 in the parental strain JHY5210 largely mimicked the LA tolerance and LA production properties of strain JHY5310. Gsf2 is known to be necessary for proper localization of certain hexose transporters including Hxt1 in the plasma membrane. Therefore, deletion of GSF2 leads to reduced glucose uptake rate, thereby alleviating glucose repression. S. cerevisiae is a Crabtree positive strain having a strong tendency of catabolizing glucose via ethanol fermentation even under aerobic conditions.
Therefore, targets of glucose repression include not only gluconeogenesis and utilization of alternative carbon sources, but also respiration\(^27,29\). We confirmed that \(\text{GSF2}^-\) deletion derepressed glucose-repressed genes including genes involved in respiratory pathway. More efficient ATP synthesis and NAD\(^+\) regeneration by respiration might rescue the growth defects of JHY5210. Considering the fact that ATP depletion via intensive export of protons and lactate anions is one of the major factors inhibiting cell growth in LA-producing cells\(^5,6,8,10,11\), more efficient ATP synthesis via respiration might contribute to the LA tolerance in JHY5212 lacking \(\text{GSF2}^-\).

However, \(\text{GSF2}^-\) deletion in wild-type strain led to an opposite effect of increasing LA sensitivity, which was commonly observed in other glucose derepressed mutants such as \(\text{mig1}^-\) and \(\text{hxk2}^-\). Therefore, unlike in JHY5210 strain, LA-dependent depletion of ATP might play a minor role in LA toxicity in wild type. LA-dependent depletion of ATP might be more detrimental for cells with impaired energy metabolism. Instead, increasing respiration by glucose derepression seems to increase LA sensitivity in wild-type background, which might be related to the fact that LA can induce oxidative stress, which is linked to the generation of reactive oxygen species (ROS) during the respiratory metabolism\(^18,36\). These results are consistent with a previous report showing higher LA toxicity under aerobic conditions than anaerobic conditions\(^15\).

In \(S.\ cerevisiae\), glucose uptake is facilitated by hexose transporters (HXTs). Hxt1 to Hxt7, in the plasma membrane\(^30\). HXTs, having different glucose affinities, are differentially expressed depending on extracellular glucose concentrations by complex regulatory networks involving both glucose induction and glucose repression mechanisms\(^30\). Glucose induction is mainly mediated by plasma membrane glucose sensors, Snf3 and Rgt2, and transcriptional repressor Rgt1\(^37\), whereas Snf1 kinase and Mig1 repressor plays a central role in glucose repression\(^27,29,38\). In the absence of glucose, expression of \(\text{HXT1, HXT2, HXT3, and HXT4}\) genes encoding low to medium-affinity \((K_m\approx5–100\ \text{mM})\) glucose transporters are repressed by Rgt1 in association with co-repressors Mth1 and Std1\(^27,39\). The presence of extracellular glucose was sensed by Snf3 and Rgt2, leading to the degradation of Mth1 and Std1, which then resulted in derepression of \(\text{HXT}\) genes\(^38\). In addition, Mig1 represses \(\text{MTH1}\) expression under high-glucose conditions, reinforcing the inactivation of Mth1 by glucose\(^37\). On the other hand,

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**Figure 5. Improvement of D-LA production by deleting \(\text{HXK2}\) or \(\text{MIG1}\) involved in glucose repression.**

JHY5210 and JHY5210-derived deletion strains were cultured in YPD medium containing 50 g/L glucose. Cell growth (a), residual glucose concentration (b), production levels of D-LA (c) and ethanol (d) were monitored. Error bars indicate standard deviations of three independent experiments.
expression of HXT1, HXT2, HXT3, and HXT4 as well as HXT6 and HXT7 encoding high-affinity (K_m ~ 1 mM) glucose transporters is repressed by Mig1 in the presence of glucose. Mig1 also represses other glucose-repressed genes in association with Hxk2. Hxk2 has a dual role as a major hexokinase under high-glucose conditions and as an intracellular glucose sensor positively regulating glucose repression. Upon glucose depletion, Snf1 is activated, which in turn inactivates Mig1 and activates transcription factors such as Cat8, Sip4, and Adr1, resulting in expression of genes involved in the utilization of alternative carbon sources, gluconeogenesis, glyoxylate cycle, and other metabolic pathways.
and respiration\textsuperscript{29,38}. We showed that alleviating glucose repression by GSF2 deletion is more effective in increasing LA tolerance than the deletion of MIG1 or HXK2. These results might be in part related to the fact that Mig1 and Hxk2 regulate only a subset of glucose repressed genes, whereas the decrease in glucose uptake rate in gsf2Δ might trigger wider range of cellular responses.

Although rapid glucose uptake by efficient ethanol fermentation might provide a selective advantage for yeast cells living in natural environments, glucose repression of the respiratory pathway might be undesirable for some industrial applications. For example, in the case of producing biomass-directed products such as cell itself or proteins, it might be useful to shift the metabolic flux from fermentation to respiration, which can provide higher biomass yield on glucose\textsuperscript{32,41}. On the other hand, to maximize metabolite production, it is desirable to minimize biomass yield. However, depending on the properties of target chemicals and engineered biosynthetic pathways, increasing respiration capacity by alleviating glucose repression can be advantageous to improve production. In this study, we demonstrated one such example that more efficient generation of ATP and NAD\textsuperscript{+} via respiration could rescue the growth defects of LA-producing cells. In addition, more efficient regenerating NAD\textsuperscript{+} through respiratory pathway could be desirable for engineered strains having redox imbalance. For example, relieving glucose repression restored the growth defects of \textit{S. cerevisiae} strain lacking pyruvate decarboxylase (PDC)\textsuperscript{42–44}. PDC catalyzes the conversion of pyruvate to acetaldehyde, the first step in the ethanol production pathway. Therefore, PDC-negative strain (pdc1Δ, pdc5Δ, and pdc6Δ) could be useful as a platform strain to produce pyruvate-derived products, but its practical applications are limited due to severe growth defects in high glucose and requirement of C2 such as ethanol and acetate\textsuperscript{45}. Evolved PDC-negative strains developed by three different groups revealed mutations in the same gene, MTH1\textsuperscript{46–48}. The identified Mhl1 mutants are supposed to be resistant to glucose-dependent degradation, resulting in reduced glucose influx by repressing HXT genes. In the case of PDC-negative strain, glucose derepression might rescue the growth defects by preventing intracellular accumulation of pyruvate to toxic levels and by regeneration of NAD\textsuperscript{+} via respiration\textsuperscript{45}. Introduction of the MTH1 mutant genes into the PDC-negative strain has been applied to produce pyruvate, LA, and 2,3-butanediol\textsuperscript{46,47}.

Impaired plasma membrane localization of Hxt1, the major HXT expressed under high-glucose conditions, might be the main reason for the glucose derepression phenotype of GSF2 deletion strain, but proper localization of other membrane proteins might also require Gsf2 function\textsuperscript{21}. Therefore, we cannot rule out the possibility that impaired localization of other Gsf2 target proteins might also contribute to the LA tolerance of GSF2 deletion strain. However, since deletion of GSF2 has been proven to be effective in inducing glucose derepression in different strain backgrounds including wild type, it could be applied to various applications where increasing biomass yield or respiratory flux is desirable. In addition to producing biomass-directed products, GSF2 deletion would be useful if the production of target chemicals requires efficient ATP production or NAD\textsuperscript{+} regeneration, and also for the production of TCA cycle intermediates such as succinic acid and fumaric acid.

**Methods**

### Strains and media and culture conditions.

All yeast strains and primers used in this study are listed in Table 2 and Supplementary Table S1, respectively. \textit{S. cerevisiae} CEN.PK2-1C strain (MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2) was used as a parental strain. Deletion strains were generated by PCR-mediated homologous recombination based on Cre/loxP recombination system\textsuperscript{49}. Deletion cassettes were obtained by PCR amplification from pUG27 or pUG72 plasmid using target gene-specific primer pairs (d\_GENE F and d\_GENE R) and then introduced into \textit{S. cerevisiae} strains. Correct integration of the cassette was confirmed by PCR analysis using confirmation primer pairs (c\_GENE F and c\_GENE R). All yeast cells were cultured in YPD medium (20 g/L peptone, 10 g/L yeast extract, and 20 or 50 g/L glucose) or synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids, 20 or 50 g/L glucose, 1.67 g/L amino acids dropout mixture lacking His, Trp, Leu, and Ura) supplemented with auxotrophic amino acids as required. For LA production experiments, \textit{OD}_{600} of 1 of pre-cultured yeast strains were harvested and resuspended in 5 mL of YPD or SC-Ura medium containing 50 g/L glucose. Yeast cells were cultured in a 50 mL screw cap conical tube at 30 °C with shaking at 170 rpm.

### Plasmids.

Plasmids used in this study are listed in Table 2. GSF2, SYN8, STM1, and SIF2 ORFs were amplified by PCR from CEN.PK2-1C genomic DNA, and then cloned between BamHI and SalI sites of p416GPD plasmid.

### Whole genome sequencing analysis.

The unevolved strain JHY5210 and the evolved strain JHY5310 were cultured and harvested for the genomic DNA extraction. All genomic DNA were isolated using HiGenEx\textsuperscript{TM} Genomic DNA Prep Kit for Yeast (BIOFACT, Korea). The DNA library was prepared using the TruSeq DNA sample preparation kits (Illumina, USA) and sequenced using Illumina HiSeq-2000 (Illumina, USA) at 2 × 75 bp read pairs. The raw reads were processed with Trimmomatic (version 0.3) to remove adapters and poor quality reads, and then reads shorter than 36 bp were discarded. The filtered reads were mapped to the reference genome (CEN.PK 113-7D, http://cenpk.tudelft.nl) using Burrows-Wheeler Aligner (BWA) software (ver. 0.7.1). Potential PCR duplicates were removed with the MarkDuplicates program of the Picard package (http://picard.sourceforge.net). Indels were located and realigned with Realigner Target Creator/Indel Realigner of Genome Analysis Toolkit (GATK), and detected using VarScan software (ver. 2.3.7). Single nucleotide variants (SNVs) were detected using MuTect (ver 1.1.7).

### Quantitative reverse transcription PCR (qRT-PCR) analysis.

\textit{OD}_{600} of 0.5 of JHY5210 and JHY5212 strains were cultured in 10 ml YPD medium containing 50 g/L glucose in a 100 ml flask at 30 °C for 47 h. Total RNA from each harvested cells was isolated using the hot acidic phenol RNA extraction method\textsuperscript{49}, and then the relative amount of mRNA was determined by qRT-PCR\textsuperscript{50} using a LightCycler 480 II instrument (Roche Diagnostics, Germany) with SYBR green PCR master mix (Roche Diagnostics, Germany). The \textit{ACT1} gene was
used as a control to normalize the transcription level of target genes. Primer sequences used for qRT-PCR are listed in Supplementary Table S1.

**Analytical methods.** Samples collected from culture supernatant were filtered using a 0.22 μm syringe filter before detecting metabolites. To quantify the concentration of ethanol, glucose and lactate, high performance liquid chromatography (HPLC) analysis was performed in UltiMate 3000 HPLC system (Thermo Fisher Scientific) equipped with Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm, 5 μm) at 60 °C with 5 mM H2SO4 at a flow rate of 0.6 mL/min. Refractive index (RI) was used as a detector keeping at 35 °C. Growth was monitored by determining optical density at 600 nm using spectrophotometer (Varian Cary50 UV/Vis spectrophotometer, Agilent) as described previously. Specific rates of glucose consumption and ethanol formation (g: mmol·h⁻¹·g⁻¹ of dry biomass) in the exponential growth phase were calculated from three independent experiments. Dry cell weight (DCW) was estimated by multiplying optical density value and a conversion factor (0.3) together.

**Table 2. Yeast strains and plasmids used in this study.**

| Strain / Plasmid | Genotype and description | Reference |
|------------------|--------------------------|-----------|
| **S. cerevisiae** strains |
| CEN.PK2-1C | MATα ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2 | EUROSCARF |
| JHY5101 | CEN.PK2-1C gpd1Δ::loxP-URA3-loxP | This study |
| JHY5102 | CEN.PK2-1C mig1Δ::loxP-URA3-loxP | This study |
| JHY5103 | CEN.PK2-1C hsh2Δ::loxP-his5Δ::loxP | This study |
| JHY5160 | CEN.PK2-1C did1Δ::loxP jen1Δ::loxP adh1Δ::loxP gpd1Δ::loxP gpd2Δ::loxP | 16 |
| JHY5210 | JHY5160 pdc1Δ::P Pays/Lm ldhA-TGPD | 16 |
| JHY5212 | JHY5210 gfpΔ::loxP-his5Δ::loxP | This study |
| JHY5213 | JHY5210 syn8Δ::loxP-his5Δ::loxP | This study |
| JHY5214 | JHY5210 stu1Δ::loxP-his5Δ::loxP | This study |
| JHY5215 | JHY5210 sti2Δ::loxP-his5Δ::loxP | This study |
| JHY5216 | JHY5210 mig1Δ::loxP-his5Δ::loxP | This study |
| JHY5217 | JHY5210 lex2Δ::loxP-his5Δ::loxP | This study |
| JHY5310 | Evolved strain from JHY5210 | 16 |
| JHY602 | CEN.PK2-1C adh1Δ::loxP adh2Δ::loxP adh3Δ::loxP adh4Δ::loxP adh5Δ::loxP | 52 |
| JHY604 | JHY602 gpd1Δ::loxP-URA3-loxP | This study |
| JHY604 | CEN.PK2-1C adh1Δ::loxP gpd1Δ::loxP gpd2Δ::loxP | 52 |
| JHY604 | JHY604 gpd2Δ::loxP-URA3-loxP | This study |
| **Plasmids** |
| pUG7 | Plasmid containing loxP-his5Δ::loxP deletion cassette | EUROSCARF |
| pUG2 | Plasmid containing loxP-URA3-loxP deletion cassette | EUROSCARF |
| p416GPD | CEN/ARS plasmid, URA3, Pura3-TEF1-GPD-TGPD | 53 |
| p416GPD-GSF2 | CEN/ARS plasmid, URA3, Pura3-TEF1-GSF2-TGPD | This study |
| p416GPD-SYN8 | CEN/ARS plasmid, URA3, Pura3-TEF1-SYN8-TGPD | This study |
| p416GPD-STM1 | CEN/ARS plasmid, URA3, Pura3-TEF1-STM1-TGPD | This study |
| p416GPD-SIF2 | CEN/ARS plasmid, URA3, Pura3-TEF1-SIF2-TGPD | This study |

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Acknowledgements
This work was supported by the Ministry of Trade, Industry & Energy (MOTIE, Korea) under Industrial Technology Innovation Program (No. 10062248) and by C1 Gas Refinery Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2016M3D3A1A01913245).

Author Contributions
S.-H.B. and J.-S.H. designed the experiments and wrote the manuscript. S.-H.B., E.Y.K. and S.-Y.K. performed the experiments and analyzed the data. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Baek, S.-H. et al. GSF2 deletion increases lactic acid production by alleviating glucose repression in Saccharomyces cerevisiae. Sci. Rep. 6, 34812; doi: 10.1038/srep34812 (2016).

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