Gene amplification on demand accelerates cellobiose utilization in engineered *Saccharomyces cerevisiae*

Eun Joong Oh[1,2], Jeffrey M. Skerker[3,4], Soo Rin Kim[5], Na Wei[6], Timothy L. Turner[1,2], Matthew J. Maurer[3,4], Adam P. Arkin[3,4] and Yong-Su Jin[1,2]

[1]Department of Food Science and Human Nutrition, [2] Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

[3]Department of Bioengineering, University of California at Berkeley, Berkeley, CA 94720, USA

[4]Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

[5]School of Food Science and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea

[6]Department of Civil and Environmental Engineering and Earth Sciences, University of Notre Dame, Notre Dame, 46556, USA

Correspondence should be addressed to Yong-Su Jin (ysjin@illinois.edu).

Prepared for submission to *AEM*

Abstract

Efficient microbial utilization of cellulosic sugars is essential for the economic production of biofuels and chemicals. Although *Saccharomyces cerevisiae* is a robust microbial platform widely used in ethanol plants using sugar cane and corn starch in large-scale operations, glucose repression is one of the significant barriers to the efficient fermentation of cellulosic sugar mixtures. A recent study demonstrated that intracellular utilization of cellobiose by engineered yeast expressing a cellobiose transporter (*cdt-1*) and an intracellular β-glucosidase (*gh1-1*) can alleviate glucose repression, resulting in simultaneous co-fermentation of cellobiose and non-glucose sugars. Here we report enhanced cellobiose fermentation by engineered yeast expressing *cdt-1* and *gh1-1* through laboratory evolution. When *cdt-1* and *gh1-1* were integrated into the genome of yeast, the single copy integrant showed a slow cellobiose consumption rate. However, cellobiose fermentation rates by engineered yeast increased gradually during serial subcultures on cellobiose. Finally, an evolved strain exhibited a 15-fold higher cellobiose fermentation rate. To identify responsible mutations in the evolved strain, genome sequencing was performed. Interestingly, no mutations affecting cellobiose fermentation were identified, but the evolved strain contained nine copies of *cdt-1* and 23 copies of *gh1-1*. We also traced the copy numbers of *cdt-1* and *gh1-1* of mixed populations during the serial subcultures. The copy numbers of *cdt-1* and *gh1-1* in the cultures increased gradually with similar ratios as cellobiose fermentation rates of the cultures increased. These results suggest that the cellobiose assimilation pathway (transport and hydrolysis) might be a rate-limiting step in engineered yeast, and copies of genes coding for metabolic enzymes might be amplified in yeast if there is a growth advantage. This study presents that on-demand gene amplification might be an efficient strategy for yeast metabolic engineering.

Importance

In order to enable rapid and efficient fermentation of cellulosic hydrolysates by engineered yeast, we delve into the limiting factors of cellobiose fermentation by engineered yeast expressing a cellobiose transporter (*cdt-1*) and an intracellular β-glucosidase (*gh1-1*). Through laboratory evolution, we isolate mutant strains capable of fermenting cellobiose much faster than a parental strain. Genome sequencing of the fast cellobiose-fermenting mutant reveals that there are massive amplifications of *cdt-1* and *gh1-1* in the yeast genome. We also find positive and quantitative relationships between the rates of cellobiose consumption and the copy numbers of *cdt-1* and *gh1-1* in the evolved strains. Our results suggest that the cellobiose assimilation pathway (transport and hydrolysis) might be a rate-limiting step for efficient cellobiose fermentation. We demonstrate not only the feasibility of optimizing heterologous metabolic pathways in yeast through laboratory evolution, but also on-demand gene amplification in yeast which can be broadly applicable for metabolic engineering.

Introduction

The efficient utilization of sugars prevalent in cellulosic hydrolysates by microorganisms is essential to develop a bioeconomy through the cost-effective production of biofuels and value-added chemicals (1). Sugarcane and corn starch have been used as sugar sources for value-added biotransformation based on microbial fermentation, but the "food versus biofuel" issue has been a concern (2). Cellulosic biomass could provide an ethical and sustainable solution (3). Sugarcane and starch contain only hexoses, but cellulosic biomass consists of various hexoses and pentoses. Glucose and xylose are the primary sugars in cellulosic hydrolysates (4). Therefore, efficient microbial fermentation of both hexose and pentose sugars is necessary to produce biofuels such as ethanol (5).

The hexose-fermenting *Saccharomyces cerevisiae* has been employed for the large-scale fermentation of sugarcane and corn starch, as well as for many other industrial fermentations for the production of value-added chemicals (6, 7). In addition, genetic tools of *S. cerevisiae* have been well-developed so that sophisticated genetic modifications can be readily performed (8). Nonetheless, *S. cerevisiae* cannot natively ferment xylose, which is the second most abundant sugar in cellulosic hydrolysates. Therefore, metabolic engineering efforts to construct xylose-fermenting *S. cerevisiae* have been made for over two decades (9). A common strategy for the construction is to introduce either the oxidoreductase pathway consisting of xylose reductase (XR) and xylitol dehydrogenase (XDH), or the single-step isomerization pathway catalyzed by xylose isomerase (XI) (10-17). After introducing the xylose assimilation pathways, engineered *S. cerevisiae* was able to convert xylose into ethanol but utilized glucose preferentially from mixtures of glucose and xylose (18, 19). This "glucose repression" problem is a major barrier to efficient fermentation using sugar mixtures from terrestrial and marine biomass (20, 21). To mitigate this issue, direct fermentation of cellobiose, a dimer of glucose, by engineered yeast has been proposed (22, 23). Wild-type *S.*

cerevisiae cannot utilize cellobiose, but the introduction of a cellodextrin transporter gene (*cdt-1*) and an intracellular β-glucosidase gene (*gh1-1*) from *Neurospora crassa* allowed cellobiose assimilation in *S. cerevisiae* (23). The extracellular glucose can inhibit xylose uptake, while the intracellular glucose does not impede xylose transport. As cellobiose is hydrolyzed into glucose intracellularly in engineered yeast, glucose repression on xylose can be alleviated so that engineered yeast with both xylose and cellobiose-assimilating pathways was able to ferment cellobiose and xylose simultaneously. Moreover, the simultaneous co-fermentation of cellobiose and xylose led to a higher ethanol yield and productivity as compared to the sequential fermentation of glucose and xylose (22).

There are two potential problems with cellobiose fermentation in prior studies (22, 24). First, the engineered yeast contained the episomal plasmids that can result in plasmid loss in non-selective media due to a metabolic burden on the host (25). Second, the engineered yeast accumulated substantial amounts of extracellular cellodextrin due to the transglycosylation activity of β-glucosidase when there is a mismatch between glucose production and consumption rates during the cellobiose fermentation. While the accumulated cellodextrin can be re-assimilated later, it might decrease ethanol productivity (22). In this study, we aimed to solve these two problems in the cellobiose-fermenting yeast constructed using episomal plasmids. To alleviate these issues, we integrated the cellobiose assimilation pathway into the genome and performed laboratory evolution using cellobiose as the sole carbon source. When we introduced the *cdt-1* and *gh1-1* genes into the genome of *S. cerevisiae*, the engineered strain showed a low cellobiose consumption rate. However, we observed that the cellobiose fermentation rate increased significantly after serial subcultures on cellobiose. After isolating the evolved strains, we identified the genetic changes responsible for the improved phenotype of the evolved strain through genome sequencing. Interestingly, the improved cellobiose fermentation with reduced cellodextrin accumulation

was not caused by mutations in endogenous genes of *S. cerevisiae*, but instead greatly increased copy numbers of *cdt-1* and *gh1-1* with a presumably optimized copy-number ratio of 1:2, respectively. These results suggest that copies of genes coding for metabolic enzymes might be amplified in yeast if there is a growth advantage, and the cellobiose assimilation pathway (transport and hydrolysis) might be a rate-limiting step of cellobiose fermentation by engineered *S. cerevisiae*.

Materials and Methods

Strains, media and culture conditions

The yeast plasmids and strains used in this study are summarized in Table 1. *Escherichia coli* DH5 (*F– recA1 endA1 hsdR17 [rK– mK+] supE44 thi-1 gyrA relA1*) (Invitrogen, Gaithersburg, MD) was used for gene cloning and manipulation. The *E. coli* was grown in Luria-Bertani medium. 50 µg/mL of ampicillin was added as required. Yeast strains were routinely cultivated at 30 °C in YP medium (10 g/L of yeast extract and 20 g/L of peptone) with 20 g/L of glucose or 20 g/L of cellobiose. To select the transformants using an auxotrophic marker, yeast synthetic complete (YSC) medium was used, which contained 6.7 g/L of Yeast Nitrogen Base (YNB) plus 20 g/L of glucose, 20 g/L of agar, and CSM-Leu-Trp-Ura-His (MP Biomedicals, Santa Ana, CA) with a supply of appropriate nucleotides and amino acids.

Strains and plasmid construction

All of the primers used for constructing plasmids are listed in Table S1. We used the CloneEZ PCR cloning Kit (Genscript, Piscataway, NJ) to construct plasmids expressing β-glucosidase (*gh1-1*) and cellodextrin transporter (*cdt-1*) from *N. crassa*. The plasmids

pRS425-gh1-1 and pRS426-cdt-1 were constructed in a previous study to overexpress β-glucosidase (*gh1-1*) and cellodextrin transporter (*cdt-1*) under the control of the *PGK1* promoter and *CYC1* terminator (Galazka et al., 2010). Each expression cassette of β-glucosidase and cellodextrin transporter was amplified by PCR between the T3 and T7 sites on both pRS425-gh1-1 and pRS426-cdt-1. Linearized vectors were prepared by PCR, amplifying the backbone region (including the auxotrophic markers and replicons) between T3 and T7 primer binding sites of templates pRS405, pRS406, pRS423, pRS424, pRS425, and pRS426. The expression cassettes for the T3-T7 section were cloned to the linearized vectors by a recombination procedure. The resulting plasmids pRS405-gh1-1 and pRS406-cdt-1 were integrated into the *LEU2* and *URA3* loci of the *S. cerevisiae* D452-2 strain, yielding the EJ1 strain. The *S. cerevisiae* CEN.PK2-1D strain was used as a host strain to express β-glucosidase (*gh1-1*) and cellodextrin transporter (*cdt-1*) using multi-copy plasmids. The yeast EZ-Transformation kit (MP Biomedicals, Santa Ana, CA) was used to transform the expression cassettes. The transformants were selected on YSC agar medium containing 20 g/L of glucose. Amino acids and nucleotides were added as necessary.

To construct the *SVL3* deletion mutants, the *svl3Δ*::*KanMX4* cassette was amplified by PCR from the genomic DNA of the BY4742 *svl3Δ* strain in the Yeast Knockout Collection (Open Biosystems, Pittsburgh, PA). The PCR product was purified and integrated into the strain EJ1, and the deletion mutant was selected on YP medium (10 g/L of yeast extract and 20 g/L of peptone) containing 20 g/L of glucose with 500 μg/mL of G418. The *S. cerevisiae* *SVL3* gene was PCR amplified from the genomic DNA of strains EJ1 and EJ2 using the primers SVL3 cloning-F and SVL3 cloning-R. Each amplified DNA fragment was cloned into the plasmid pRS423 under the control of the *TDH3* promoter and *CYC1* terminator. The transformation of the constructed plasmids was performed using the yeast EZ-Transformation kit (MP Biomedicals, Santa Ana, CA).

176

177 **Fermentation experiments**

178 The yeast cells were grown in YP medium containing 20 g/L of cellobiose to prepare
179 inoculums for the fermentation experiments. The cells were harvested in the mid-exponential
180 phase (OD_{600} = 1.0) and inoculated into the experimental flasks after washing them twice
181 with sterilized water. The 125 mL flasks fermentation contained 25 mL of YP medium with
182 the appropriate sugars at 30 °C with initial optical densities (OD) at 600 nm of 1.0 or 0.1
183 under oxygen-limited conditions.

184

185 **Preparation of crude extract and β-glucosidase activity assay**

186 Yeast cells grown to mid-log phase (OD_{600} = 1.0) at 30°C in YP medium with 20 g/L
187 of cellobiose were harvested by centrifugation at 3000 × g for 5 min. The cell pellets were
188 washed and suspended in Y-PER solution (Pierce, Rockford, IL). After incubation at room
189 temperature for 20 min., the cell suspension was centrifuged at 13,000 rpm for 10 min. to
190 completely remove the cell debris. The supernatant (crude enzyme extract) was kept on ice
191 for the enzyme assay. The β-glucosidase activity was measured according to the previous
192 method (Galazka et al., 2010) in a reaction solution containing 50 mM of sodium acetate
193 buffer (pH 4.8) and 6.7 mM of para-nitrophenyl β-D-glucopyranoside (*p*NPG). The release of
194 *p*-nitrophenol (NP) was monitored by a microplate reader (Synergy 2; Biotek, Winooski, VT)
195 at 405 nm during 1 hour of incubation at 30 °C. The *p*-nitrophenol absorption coefficient was
196 18.4 $mM^{-1}cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme that
197 catalyzes 1 µmol of substrate per min. at 30 °C. The protein concentration was determined by
198 the BCA method (Pierce, Rockford, IL).

199

Quantitative PCR for determining genomic copy numbers of *gh1-1* and *cdt-1*

The genomic DNA of the strain from each subculture was prepared with the YeaStar Genomic DNA Kit (Zymo Research, Orange, CA) and quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE). Real-time PCR was performed using SYBR Green I Master (Roche) on a Lightcycler 480 instrument (Roche Applied Science, Indianapolis, IN) following the manufacturer's directions. As shown in Table S1, the primers were designed to detect the *gh1-1* and *cdt-1* genes. The concentrations of the genes were quantified by standard curves generated by the *gh1-1* and *cdt-1* fragments purified from pRS425-gh1-1 and pRS426-cdt-1 respectively. The calculations of the genomic copy numbers were determined by a comparison of the relative concentrations of the genes obtained by quantitative PCR, as described by Kim et al. (26).

Genome sequencing, single nucleotide polymorphism (SNP) discovery, and estimation of copy numbers

The genomic DNA from *S. cerevisiae* EJ1 and EJ2 was prepared using the YeaStar Genomic DNA Kit (Zymo Research, Irvine, CA). The genome sequencing was performed using an Illumina HiSeq2000 machine in the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. The barcoded shotgun genomic DNA libraries were constructed with the TruSeq DNAseq Sample Prep Kit (Illumina, San Diego, CA). The diluted equimolar libraries (10 nM) were multiplexed on a lane and sequenced using single reads for 100 nt by the Illumina HiSeq2000, with SBS chemistry version 2. The raw data were processed by Casava 1.7. SNP discovery and the estimation of the copy numbers was performed using CLC Genomics Workbench 7.0.2 (CLC Bio, Aarhus, Denmark). The sequence reads were trimmed with a quality score limit of 0.05 and assembled into reference sequences. As reference sequences, *S. cerevisiae* S288C was

used for the SNP discovery, and the combinations of the RT-PCR reference genes commonly used for quantitative gene expression (27). The read depth values for *ACT1*, *IPP1*, and *PDA1* were used to estimate the copy numbers of *gh1-1* and *cdt-1* to get the average coverage for them. Probabilistic variant detection was performed with a minimum coverage of 10 and a probability of 90, and these variants were compared to the reference variants to discover SNP.

Pulsed-field gel electrophoresis (PFGE)

Chromosomal DNA was prepared with the CHEF genomic DNA plug kit (Bio-Rad, Hercules, CA). The agarose plugs containing yeast chromosomes were loaded to a 0.8 % agarose gel in a 0.5 × TBE buffer. Bio-Rad CHEF Mapper XA pulsed field electrophoresis system was used to separate chromosomes with following conditions: a voltage of 6 V/cm, a run time of 24 hours, and an angle of 120 ° at 14 °C. The gel was stained with ethidium bromide after electrophoresis.

Analytical methods

Cell growth was monitored by OD at 600 nm using a UV-visible spectrophotometer (Biomate 5, Thermo, NY). The glucose, cellobiose, xylose, xylitol, glycerol, acetate and ethanol concentrations were determined by high-performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector using a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 0.005 N of H_2SO_4 at a flow rate of 0.6 mL/min at 50 ˚C.

Results

Construction of cellobiose-utilizing strains via integration of *cdt-1* and *gh1-1*

In contrast to prior studies that employed episomal plasmids for expressing *cdt-1* and *gh1-1*, we integrated *cdt-1* and *gh1-1* into the genome of *S. cerevisiae*. The resulting EJ1 strain harboring both *cdt-1* and *gh1-1* in the genome showed a limited ability to metabolize cellobiose as the sole carbon source (Fig. 1). When 80 g/L of cellobiose was provided, the EJ1 strain consumed 51 g/L of cellobiose and produced 16 g/L of ethanol during 120 hours, but further metabolism of cellobiose was not significant until ~300 hours. The volumetric productivity ($P_{Ethanol}$) of the cellobiose fermentation was 0.037 g/L·h and ethanol yield ($Y_{Ethanol}$) was 0.18 g ethanol/g cellobiose. In addition to the low values for $P_{Ethanol}$ and $Y_{Ethanol}$, the cellobiose consumption rate of the EJ1 strain decreased during the fermentation. The initial rate was 0.40 g/L·h and after 140 hours, when acetate concentration had increased to 1.94 g/L, it became much slower. Thus, acetate accumulation might have inhibited cellobiose consumption. When compared to a strain expressing episomal multi-copies of the same genes (24), the EJ1 strain expressing the single chromosomal copy of *cdt-1* and *gh1-1* assimilated cellobiose at an extremely slow rate (2.22 versus 0.18 g/L·h).

Improved cellobiose-fermenting capability through laboratory evolution

To improve the cellobiose fermentation rate, the EJ1 strain was subjected to laboratory evolution, which consisted of serial subcultures in YP medium containing 80 g/L of cellobiose as the sole carbon source. We hypothesized that repeated subculturing on 80 g/L of cellobiose would select for spontaneous mutants with an increased growth rate. As expected, the EJ1 strain showed gradual but noticeable improvement in its cellobiose consumption rate during the serial subcultures (Fig. 1 and Supplementary Fig. S1). The ethanol concentration was 11.6 g/L at the end of the first culture, but the ethanol production was 35.2 g/L at the end of the last culture. Both yields and productivities of ethanol production from cellobiose were improved substantially as the serial subcultures progressed

(Fig. 1). As the twelfth subculture might be a heterogenous population of various evolved strains, we plated and selected nine colonies from the last subculture, and their cellobiose fermentation capabilities were evaluated using the same culture medium (80 g/L of cellobiose) used for the serial subcultures (Supplementary Fig. S2). The colony that exhibited the highest ethanol productivity was named EJ2. The cellobiose fermentation profile by the EJ2 strain was similar to those of the last serial subculture. These results indicate that the majority of the population in the last subculture had similar phenotypes to the EJ2 strain. It was evident that the EJ2 strain can ferment cellobiose much faster than the EJ1 strain, possibly as a result of the EJ2 strain having accumulated beneficial mutations in the genome during the serial subcultures.

Identification of genetic mutations in the evolved cellobiose-fermenting strain

Laboratory evolution under a particular selection condition and subsequent genome sequence analysis of a selected colony with the desired phenotype is a proven approach of inverse metabolic engineering (26, 28). After comparing the genome sequences of the EJ2 and EJ1 strains, we identified only one non-synonymous change in the EJ2 strain as compared to the EJ1 strain. A protein of unknown function encoded by *SVL3* in the EJ2 strain had a mutation from methionine to isoleucine at position 685 (M685I) as compared to the EJ1 strain. The SNP (G2005A) in the *SVL3* gene was confirmed by Sanger sequencing of a PCR product amplified from the genomic DNA of the EJ2 strain. As a previous report discovered that *SVL3* might be involved in vacuolar function (29), we reasoned that the alteration of vacuole function might increase the stabilities of the cellobiose transporter and β-glucosidase and improve the cellobiose fermentation. To test this idea, we both deleted and overexpressed the *SVL3* gene in the slow cellobiose-fermenting strain EJ1, and examined cellobiose fermentation rates of the EJ1, the EJ1 *svl3Δ*, and the EJ1 *SVL3* overexpressing strains.

However, no changes in cellobiose fermentation by those strains were observed. In addition, we overexpressed the mutant allele of *SVL3* (M685I) that was present in EJ2, but there was no significant difference in the cellobiose fermentation rate (Supplementary Fig. S3). These results suggest that the mutation in the *SVL3* gene is not directly associated with the improved cellobiose fermentation capability of the EJ2 strain.

As there were no additional non-synonymous variants in coding regions, we then investigated the copy number variations in EJ2 as compared to EJ1. The average read depth, which is the number of short sequences mapped to each target from the Illumina sequence reads, was used to estimate the copy number of the integrated *cdt-1* and *gh1-1* genes. Therefore, we examined the average read depths of *cdt-1* and *gh1-1* and all open reading frames in EJ1 and EJ2 strains. We could not find any copy number variations of endogenous genes in *S. cerevisiae*, but 19-fold and 17-fold increases in average read depths of *cdt-1* and *gh1-1*, respectively, in the EJ2 strain as compared to the EJ1 strain were observed (Fig. 2A). We also determined the copy numbers of *cdt-1* and *gh1-1* in the EJ1 and EJ2 strains using real-time quantitative PCR (qPCR). As expected, the EJ2 strain showed much higher copy numbers for *cdt-1* and *gh1-1* (9 and 23, respectively) than for EJ1 (1 and 2, respectively) (Fig. 2A). This result suggested that the gene amplification of *cdt-1* and *gh1-1* in the genome might be responsible for the improved phenotype of the evolved strain (EJ2). We also measured the β-glucosidase activities in the EJ1 and EJ2 strains and observed that the crude extract of the EJ2 strain exhibited a higher β-glucosidase activity than the EJ1 strain, suggesting that the increased copy numbers of *gh1-1* in the EJ2 strain resulted in increased GH1-1 enzyme (Fig. 2B).

Amplification of *cdt-1* and *gh1-1* during serial subcultures on cellobiose

As we identified the increased copy numbers of *cdt-1* and *gh1-1* in the evolved EJ2

strain that is a strain isolated from the final serial subculture, we reasoned that the copy numbers of *cdt-1* and *gh1-1* in the evolving strains might increase during the serial subcultures. Interestingly, the copy numbers of *cdt-1* and *gh1-1* in the evolving strains increased gradually with good correlations of cellobiose consumption rates and ethanol yields (Fig. 1 and Fig. 3). Notably, there was a significant improvement of the cellobiose consumption rate at the fifth subculture with a substantial increase in the copy numbers of *cdt-1* and *gh1-1* (from 3 copies of *cdt-1* and 5 copies of *gh1-1* into 6 copies of *cdt-1* and 14 copies of *gh1-1*). Whereas it took more than 140 hours to consume 80 g/L of cellobiose in the initial EJ1 strain, it took only 71 hours to ferment 80 g/L of cellobiose after the fifth subculture. This suggests that amplification of the *cdt-1* and *gh1-1* genes during laboratory evolution was the cause of improved cellobiose fermentation.

The massive gene amplifications of *cdt-1* and *gh1-1* in the genome of the EJ2 strain was also confirmed by PFGE. The expression cassettes for *cdt-1* and *gh1-1* were integrated into the *URA3* and *LEU2* loci in chromosome V and III, respectively in the EJ1 strain (Fig 4A). Therefore, the sizes of chromosome V and III of the EJ2 strain should be bigger than those of the EJ1 strain. As the size of the *cdt-1* cassette was 7.3 kb and the size of the EJ2 chromosome V was ~100 kb bigger, this is consistent with a copy number of 14 (7.3 kb/copy × 14 copies) (Fig. 4B). The size of Chromosome III of the EJ2 strain appeared to be ~220 kb larger than EJ1, which is consistent with a *gh1-1* cassette copy number of 27 (8.1 kb/copy × 27 copies) (Fig. 4B). The sizes of the chromosomes in the EJ1 and EJ2 strains obtained from PFGE were entirely consistent with the copy number estimates determined by Illumina read depth analysis and qPCR. Together, all three methods demonstrate that massive gene amplification in the chromosomes occurred during laboratory evolution, and likely contributed to substantial improvements in cellobiose fermentation.

Applied and Environmental Microbiology

Effects of copy number variations of *cdt-1* and *gh1-1* on fermentation of cellobiose

To determine whether increased copy number of the *cdt-1* and *gh1-1* genes was responsible for the enhanced cellobiose fermentation we observed in the evolved strains, we constructed a series of engineered yeast strains with varied numbers of the *cdt-1* and *gh1-1* cassettes. We used a different strain background (*S. cerevisiae* CEN.PK2-1D) not only because the strain has quadruple autotrophic markers, but also to examine whether or not the copy number effect is strain independent. A total of five engineered strains were constructed that contained different numbers of CEN plasmids with either the *cdt-1* or *gh1-1* expression cassettes: CEN-C1B1 had one for the *cdt-1* expression cassette and one plasmid for the *gh1-1* expression cassette, CEN-C1B2 had one plasmid containing the *cdt-1* expression cassette and two plasmids containing the *gh1-1* expression cassette, CEN-C1B3 had one plasmid containing the *cdt-1* expression cassette and three plasmids containing the *gh1-1* expression cassette, CEN-C2B1 had two plasmids containing the *cdt-1* expression cassette and one plasmid containing the *gh1-1* expression cassette, and CEN-C3B1 had three plasmids containing the *cdt-1* expression cassette and one plasmid containing the *gh1-1* expression cassette transformed into the CEN.PK2-1D strain.

Using the above five strains, we examined the effects of varied expression levels of the cellodextrin transporter and β-glucosidase on cellobiose fermentation rates. As shown in Fig. 5, *S. cerevisiae* CEN.PK2-1D strains harboring higher copy numbers of *cdt-1* or *gh1-1* exhibited higher cellobiose consumption rates and ethanol productivity than ones containing lower copy numbers of the genes. When cultured in 80 g/L cellobiose as the sole carbon source, the cellobiose consumption rate and ethanol productivity by the CEN-C3B1 strain were 35 % and 39 % higher, respectively, than those of the CEN-C1B1 strain. However, the differences in the fermentation parameters between the CEN-C2B1 and CEN-C3B1 strains were not significant, suggesting that the CEN-C2B1 strain was already saturated with *cdt-1*

375 copies (Fig. 5). We also observed that an increase in the *gh1-1* copy number improved the
376 cellobiose fermentation. The ethanol productivity of the CEN-C1B3 strain (0.402 ± 0.009
377 g/L·h) was higher than that of the CEN-C1B1 strain (0.304 ± 0.006, $P < 0.05$), corresponding
378 to a higher (30 %) cellobiose consumption rate. These results indicate that the key
379 fermentation parameters, cellobiose consumption rate and ethanol productivity, improve by
380 increasing the copy numbers of *gh1-1* and *cdt-1*, regardless of the strain background.
381
382 **Stability of the integrative cassettes of *cdt-1* and *gh1-1* in chromosomes of the EJ2 strain**
383 It is evident that the copy number increases of the *cdt-1* or *gh1-1* genes in engineered
384 yeast, either through gene amplification, or increased episomal plasmid copy number can
385 accelerate cellobiose utilization. One obvious advantage of gene amplification over episomal
386 plasmids is long-term stability under non-selective culture conditions, which are mostly used
387 in industrial fermentations. Therefore, we examined the long-term stability of the amplified
388 cassettes of *cdt-1* and *gh1-1* without any selective pressure, i.e. without using cellobiose as a
389 carbon source. We performed ten serial transfers of the EJ2 strain in 20 g/L glucose. The cells
390 from the tenth transfer (EJ2-10) were evaluated in medium containing cellobiose as the sole
391 carbon source. Interestingly, EJ2-10 exhibited similar cellobiose consumption rate and
392 ethanol productivity to the EJ2 strain (Table 2). The EJ2-10 strain yielded a comparable
393 specific cellobiose consumption rate (0.46 ± 0.01 g cellobiose/g cell/h) than EJ2 (0.49 ± 0.00
394 g cellobiose/g cell/h). Using real-time PCR (qPCR), the copy numbers for *cdt-1* and *gh1-1* (7
395 and 17) in EJ2-10 were found to be lower than in EJ2 (9 and 23) (Fig. 6). These results
396 showed that the copy numbers of *cdt-1* and *gh1-1* in engineered yeast might be varied upon
397 culture conditions but they did not change drastically. While the ten times of subcultures on
398 glucose is an exaggerated condition, the EJ2 strain was able to keep the rapid cellobiose
399 fermenting phenotype. Here, we show that cellobiose can be used as an efficient selection

pressure for gene amplification and the amplified cassettes in the genome are stable.

4. Discussion

Production of biofuels and chemicals from mixed sugars present in the hydrolysates of marine and terrestrial biomass has been anticipated for decades, but both yields and productivities of biofuels and chemicals are not currently feasible for commercialization (30-32). Among many bottlenecks, glucose repression on utilizing non-glucose sugars is a significant problem for fermenting mixed sugars by engineered *S. cerevisiae*. Direct fermentation of cellobiose by engineered yeast opened the possibility of simultaneous utilization of cellobiose and non-glucose sugars (22, 23), and it is proven that cellobiose fermentation by engineered yeast can be applied for fermenting pretreated cellulosic biomass (33, 34).

To ferment cellobiose in cellulosic hydrolysates, it is necessary for the engineered *S. cerevisiae* to keep the plasmids for expressing the β-glucosidase and the cellodextrin transporter. Therefore, we introduced cellodextrin transporter and β-glucosidase genes into the genome of *S. cerevisiae* to enable their stable expression under non-selective conditions. Also, we applied an evolutionary engineering strategy to improve the cellobiose fermentation rate of the integrant strain. In this study, the evolved strain harbored higher copy numbers of the cellodextrin transporter gene (*cdt-1*) and β-glucosidase gene (*gh1-1*) in the genome than the parental strain did. The fast cellobiose-consuming strain showed high enzyme activity with *gh1-1*, indicating that the β-glucosidase activity corresponded to the copy number variations. The tandem amplification of genes might be initiated by the unequal crossover of the *ura3*::*URA3 PGK1p-cdt-1-CYC1t* or *leu2*::*LEU2 PGK1p-gh1-1-CYC1t* construct in the genome (Fig. 4A). This difference suggests that amplification of integrated *cdt-1* and *gh1-1* in the genome during laboratory evolution might be the primary genetic event. In a prior study,

xylose utilization by engineered *S. cerevisiae* harboring xylose isomerase (*xylA*) was also significantly improved by tandem gene amplification of the *xylA* in the chromosome, resulting in an increase in the expression level of the xylose isomerase during evolutionary engineering (12). Taken together, these findings indicate that the increase by gene amplification in the copy numbers of the essential genes for sugar utilization (*cdt-1* and *gh1-1* or *xylA*) might be a key factor in improving the fermentation performance.

The effect of the copy number variations of *cdt-1* and *gh1-1* on cellobiose fermentation in the recombinant *S. cerevisiae* was also tested using a different host strain. The increase of the *cdt-1* and *gh1-1* plasmid copy numbers in *S. cerevisiae* CEN.PK2-1D resulted in an improvement of the ethanol productivity and cellobiose consumption rate. In the case of the copy number variations of *cdt-1* with the limited, constant expression of *gh1-1* (CEN-C1B1, CEN-C2B1, and CEN-C3B1), the cellobiose consumption rate and ethanol productivity were saturated with two multi-copy plasmids expressing the *cdt-1*. This saturation suggests that there might be optimized levels for cellodextrin transporter and β-glucosidase in efficient cellobiose-fermenting *S. cerevisiae*. Interestingly, the copy-number ratio of *cdt-1* and *gh1-1* converged to 1:2 as the engineered strains evolved to ferment the cellobiose faster (Fig. 3). In another study, directed evolution using the screening of promoter libraries was applied to optimize the cellobiose-utilizing pathway. Similarly, the efficient cellobiose-utilizing strain showed a faster consumption rate at the optimized ratio of the expression levels of *cdt-1* and *gh1-1* (1:2.5) (35). Although our approach for improving the cellobiose fermentation was different from Yuan et al. (35), we found that the copy numbers of *cdt-1* and *gh1-1* in the engineered *S. cerevisiae* increased to the optimized ratio. These results indicated that not only the absolute increase of the copy numbers of *cdt-1* and *gh1-1* but also their ratio is important for the large improvement in cellobiose fermentation we observed in the evolved strain EJ2.

Despite the promising results from the cellobiose fermentation experiments, transient cellodextrin accumulation by the transglycosylation of β-glucosidase was observed in the medium during the processes (24). The accumulated cellodextrin might decrease the ethanol productivity because the cellodextrin transport rate and re-consumption might not be as fast as the cellobiose fermentation (22, 24). The *S. cerevisiae* D452-2 strain expressing the cellodextrin transporter and β-glucosidase from episomal plasmids (DCDT-1G) accumulated 15.5 g/L of cellodextrin in the fermentation experiments using YP medium with 80 g/L cellobiose under oxygen-limited conditions. In contrast, the EJ2 strain which is originated from the same host (*S. cerevisiae* D452-2) showed only a 2.6 g/L cellodextrin accumulation and a higher ethanol productivity (0.52 versus 1.02 g/L·h) at 24 hours under the same conditions (Supplementary Fig. S4). When the engineered yeast strains uptake cellobiose rapidly, the accumulated intracellular cellobiose be can used as a substrate for producing cellodextrin by the transglycosylation activity of β-glucosidase (*gh1-1*). It is well-known that high cellobiose concentrations cause cellodextrin production by β-glucosidase *in vitro* (36), and the intracellular cellobiose concentration is determined by the rates of cellobiose transport and hydrolysis. Optimization of the cellodextrin transporter and β-glucosidase expression levels in the genome by evolutionary engineering may minimize the accumulation of intracellular cellobiose and its transglycosylation by β-glucosidase.

By genome integration of the cellobiose-utilizing pathway followed by evolutionary engineering, we improved the cellobiose-fermentation performance of the parental strain impressively. Based on the data presented in this study, the evolved strain showed a significantly improved cellobiose consumption rate and ethanol productivity without directed evolution (35) or using an industrial strain as the host strain (37). In addition, our approach of integration followed by evolution, could be applied to construct stable systems in *S. cerevisiae* that require high gene copy number. It is hard to implement induction or antibiotic

systems in industrial fermentation processes; therefore, stable strains based on chromosomal integration are desirable (38-41). Here we used cellobiose, a sugar derived from cellulosic biomass, as a strong selective pressure to produce massive gene amplification. Since the approach did not utilize antibiotics as a selective pressure for gene amplification or need additional genetic manipulations to keep the amplified gene copy numbers after the laboratory evolution (42), it demonstrates an industrially applicable and efficient metabolic engineering strategy. Also, we observed that loss in the copy numbers of *cdt-1* and *gh1-1* in the genome of the EJ2 strain was marginal after serial subcultures on glucose for 240 hours (Fig. 6 and Table 2). The results suggested that the stability of integrated cassettes of *cdt-1* and *gh1-1* in chromosomes can be maintained even in the absence of the selection pressure. We envision that the construction of a stable industrial strain using our engineering strategy that can co-ferment cellobiose and xylose will enable a commercially viable Simultaneous Saccharification and Co-Fermentation (SSCF) process.

Acknowledgements

This work was supported by funding from the Energy Biosciences Institute.

References

1. **Wyman CE.** 2007. What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol **25:**153-157.
2. **Pimentel D, Marklein A, Toth MA, Karpoff MN, Paul GS, McCormack R, Kyriazis J, Krueger T.** 2009. Food versus biofuels: environmental and economic costs. Hum Ecol **37:**1-12.
3. **Schmer MR, Vogel KP, Mitchell RB, Perrin RK.** 2008. Net energy of cellulosic ethanol from switchgrass. Proc Natl Acad Sci U S A **105:**464-469.
4. **Kim SR, Ha SJ, Wei N, Oh EJ, Jin YS.** 2012. Simultaneous co-fermentation of mixed sugars: a promising strategy for producing cellulosic ethanol. Trends Biotechnol **30:**274-282.
5. **Rubin EM.** 2008. Genomics of cellulosic biofuels. Nature **454:**841-845.
6. **Romanos MA, Scorer CA, Clare JJ.** 1992. Foreign gene expression in yeast: a review. Yeast **8:**423-488.
7. **Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT.** 2005. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res **5:**925-934.
8. **Forsburg SL.** 2001. The art and design of genetic screens: yeast. Nat Rev Genet **2:**659-668.
9. **Kötter P, Ciriacy M.** 1993. Xylose fermentation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol **38:**776-783.
10. **Jeffries TW.** 2006. Engineering yeasts for xylose metabolism. Curr Opin Biotechnol **17:**320-326.
11. **Matsushika A, Inoue H, Kodaki T, Sawayama S.** 2009. Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Appl Microbiol Biotechnol **84:**37-53.
12. **Zhou H, Cheng JS, Wang B, Fink GR, Stephanopoulos G.** 2012. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. Metab Eng **14:**611-622
13. **Kim SR, Park YC, Jin YS, Seo JH.** 2013. Strain engineering of *Saccharomyces cerevisiae* for enhanced xylose metabolism. Biotechnol Adv **31:**851-861.
14. **Kötter P, Amore R, Hollenberg CP, Ciriacy M.** 1990. Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, *XYL2*, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. Curr Genet **18:**493-500.
15. **Bruinenberg PM, de Bot PH, van Dijken JP, Scheffers WA.** 1983. The role of redox balances in the anaerobic fermentation of xylose by yeasts. Eur Journal Appl Microbiol Biotechnol **18:**287-292.
16. **Walfridsson M, Hallborn J, Penttilä M, Keränen S, Hahn-Hägerdal B.** 1995. Xylose-metabolizing *Saccharomyces cerevisiae* strains overexpressing the *TKL1* and *TAL1* genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase. Appl Environ Microbiol **61:**4184-4190.
17. **Ho NW, Chen Z, Brainard AP.** 1998. Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol **64:**1852-1859.
18. **Trumbly R.** 1992. Glucose repression in the yeast *Saccharomyces cerevisiae*. Mol Microbiol **6:**15-21.
19. **Carlson M.** 1999. Glucose repression in yeast. Curr Opin Microbiol **2:**202-207.
20. **Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-**

Grauslund MF. 2007. Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol **74:**937-953.

21. **Wei N, Quarterman J, Jin YS.** 2013. Marine macroalgae: an untapped resource for producing fuels and chemicals. Trends Biotechnol **31:**70-77.
22. **Ha SJ, Galazka JM, Kim SR, Choi JH, Yang X, Seo JH, Glass NL, Cate JH, Jin YS.** 2011. Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. Proc Natl Acad Sci U S A **108:**504-509.
23. **Galazka JM, Tian C, Beeson WT, Martinez B, Glass NL, Cate JH.** 2010. Cellodextrin transport in yeast for improved biofuel production. Science **330:**84-86.
24. **Kim H, Lee WH, Galazka JM, Cate JH, Jin YS.** 2014. Analysis of cellodextrin transporters from *Neurospora crassa* in *Saccharomyces cerevisiae* for cellobiose fermentation. Appl Microbiol Biotechnol **98:**1087-1094.
25. **Zhang Z, Moo-Young M, Chisti Y.** 1996. Plasmid stability in recombinant *Saccharomyces cerevisiae*. Biotechnol Adv **14:**401-435.
26. **Kim SR, Skerker JM, Kang W, Lesmana A, Wei N, Arkin AP, Jin YS.** 2013. Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. PloS One **8:**e57048.
27. **Teste MA, Duquenne M, François J, Parrou JL.** 2009. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. BMC Mol Biol **10:**99.
28. **Oud B, Maris AJ, Daran JM, Pronk JT.** 2012. Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. FEMS Yeast Res **12:**183-196.
29. **Zheng B, Wu JN, Schober W, Lewis DE, Vida T.** 1998. Isolation of yeast mutants defective for localization of vacuolar vital dyes. Proc Natl Acad Sci U S A **95:**11721-11726.
30. **Fortman J, Chhabra S, Mukhopadhyay A, Chou H, Lee TS, Steen E, Keasling JD.** 2008. Biofuel alternatives to ethanol: pumping the microbial well. Trends Biotechnol **26:**375-381.
31. **Dellomonaco C, Fava F, Gonzalez R.** 2010. The path to next generation biofuels: successes and challenges in the era of synthetic biology. Microb Cell Fact **9:**1-15.
32. **Young E, Lee SM, Alper H.** 2010. Optimizing pentose utilization in yeast: the need for novel tools and approaches. Biotechnol Biofuels **3:**24-24.
33. **Lee WH, Nan H, Kim HJ, Jin YS.** 2013. Simultaneous saccharification and fermentation by engineered *Saccharomyces cerevisiae* without supplementing extracellular β-glucosidase. J Biotechnol **167:**316-322.
34. **Jin YS, Cate JH.** 2012. Model-guided strain improvement: simultaneous hydrolysis and co-fermentation of cellulosic sugars. Biotechnol J **7:**328-329.
35. **Yuan Y, Zhao H.** 2013. Directed evolution of a highly efficient cellobiose utilizing pathway in an industrial *Saccharomyces cerevisiae* strain. Biotechnol Bioeng. **110:**2874-2881.
36. **Bohlin C, Praestgaard E, Baumann MJ, Borch K, Praestgaard J, Monrad RN, Westh P.** 2013. A comparative study of hydrolysis and transglycosylation activities of fungal β-glucosidases. Appl Microbiol Biotechnol **97:**159-169.
37. **Eriksen DT, Hsieh PCH, Lynn P, Zhao H.** 2013. Directed evolution of a cellobiose utilization pathway in *Saccharomyces cerevisiae* by simultaneously engineering multiple proteins. Microb Cell Fact **12:**61.
38. **Neubauer P, Lin H, Mathiszik B.** 2003. Metabolic load of recombinant protein

production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in *Escherichia coli*. Biotechnol Bioeng **83:**53-64.

39. **Westfall PJ, Gardner TS.** 2011. Industrial fermentation of renewable diesel fuels. Curr Opin Biotechnol **22:**344-350.

40. **Olmstead J, Wallinga D.** 2010. Antimicrobial alternatives: public health risks call into question continued antibiotic use in ethanol production. Foodborne Pathog Dis **7:**871-871.

41. **Rich JO, Leathers TD, Nunnally MS, Bischoff KM.** 2011. Rapid evaluation of the antibiotic susceptibility of fuel ethanol contaminant biofilms. Bioresour Technol **102:**1124-1130.

42. **Tyo KE, Ajikumar PK, Stephanopoulos G.** 2009. Stabilized gene duplication enables long-term selection-free heterologous pathway expression. Nat Biotechnol **27:**760-765.

43. **Hosaka K, Nikawa J, Kodaki T, Yamashita S.** 1992. A dominant mutation that alters the regulation of *INO1* expression in *Saccharomyces cerevisiae*. J Biochem **111:**352-358.

44. **Entian KD, Kötter P.** 1998. 23 Yeast Mutant and Plasmid Collections. Methods Microbiol **26:**431-449.

45. **Sikorski RS, Hieter P.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19-27.

46. **Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:**119-122.

Figure legends

Figure 1. Improvement of cellobiose fermentation capability of engineered *S. cerevisiae* EJ1 during adaptive evolution on a high concentration of cellobiose. The subcultures were performed under oxygen-limited conditions (100 rpm) in YP medium with 80 g/L of cellobiose. The initial cell density of each subculture was adjusted to 0.29 g/L (OD_{600} = 1), and the cells were transferred to fresh medium when they reached the stationary phase. Symbols: biomass (open circle), cellobiose (solid square), ethanol (solid diamond), and acetate (solid triangle).

Figure 2. Copy numbers of cellodextrin transporter gene (*cdt-1*) and β-glucosidase gene (*gh1-1*) in the parental (EJ1) and evolved strains (EJ2). (A) Copy numbers of *cdt-1* and *gh1-1* estimated by genome sequencing data and qPCR. (B) The β-glucosidase activity of engineered *S. cerevisiae* EJ1 and evolved strain EJ2. The results are the means of triplicate experiments and the error bars indicate standard deviations. Cells grown to mid-log phase in YP medium containing cellobiose (20 g/L) were harvested for the enzymatic assay.

Figure 3. A gradual increase in the copy numbers of cellodextrin transporter gene (*cdt-1*) and β-glucosidase gene (*gh1-1*) during serial subcultures of EJ1. Copy numbers of *cdt-1* and *gh1-1* in each transfer were estimated by qPCR. The results are the means of triplicate experiments and the error bars indicate standard deviations.

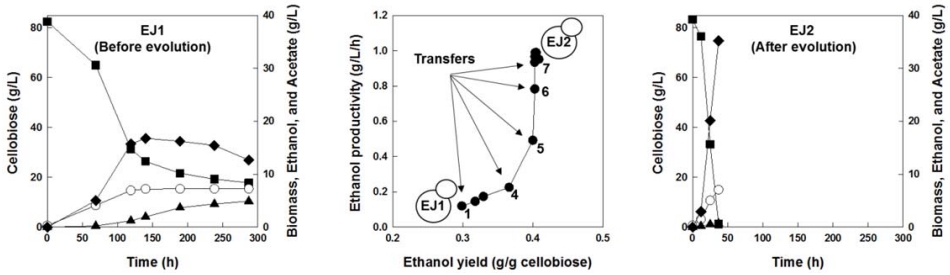
Figure 4. Amplification of integrative cassettes for *cdt-1* and *gh1-1* in chromosomes. (A) Locations of expression cassettes in chromosomes of EJ1 and EJ2. (B) Pulsed-field gel electropherogram of chromosomes from parental (D452-2) and engineered strains (EJ1 and

EJ2). The chromosome numbers and sizes are labeled on the left side and right side of the figure. M, yeast chromosome markers; Solid arrow, chromosome III; open arrow, chromosome V.

Figure 5. Effect of copy number variations of *cdt-1* and *gh1-1* in episomal plasmids on cellobiose fermentation capability of engineered *S. cerevisiae* CEN.PK2-1D. The effects of increasing episomal plasmids with *cdt-1* (CEN-C2B1 and CEN-C3B1; solid arrow) and *gh1-1* (CEN-C1B2 and CEN-C1B3; dotted arrow) in YP medium containing 80 g/L of cellobiose were compared to engineered *S. cerevisiae* CEN-C1B1. The initial cell density was adjusted to 0.029 g/L, and all fermentations were performed under oxygen-limited conditions. The results are the means of duplicate experiments and the error bars indicate standard deviations.

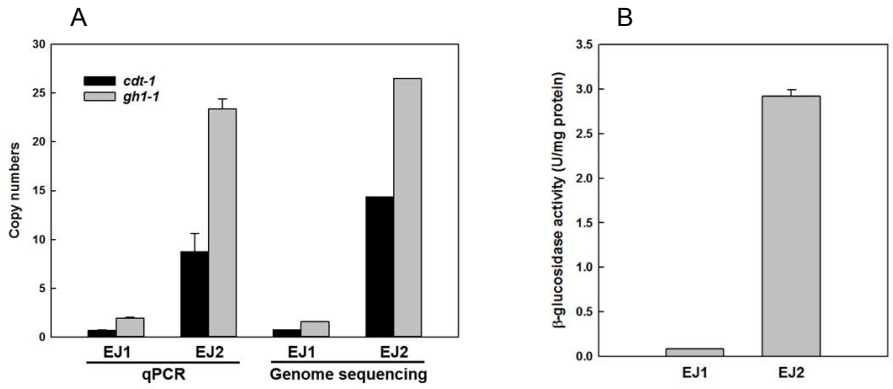
Figure 6. Changes in copy numbers of cellodextrin transporter gene (*cdt-1*) and β-glucosidase gene (*gh1-1*) during serial subcultures of EJ2 on glucose. The results are the means of triplicate experiments and the error bars indicate standard deviations. The subcultures were performed under oxygen-limited conditions (100 rpm) in YP medium with 20 g/L of glucose. The initial cell density of each subculture was adjusted to 0.29 g/L (OD_{600} = 1), and the cells were transferred to fresh medium when the cells reached the stationary phase. EJ2-10, cells from the tenth transfer.

Figure 1.



693 Figure 2.

694



695

696

697

698

699

700

701

702

703

704

705

706

707

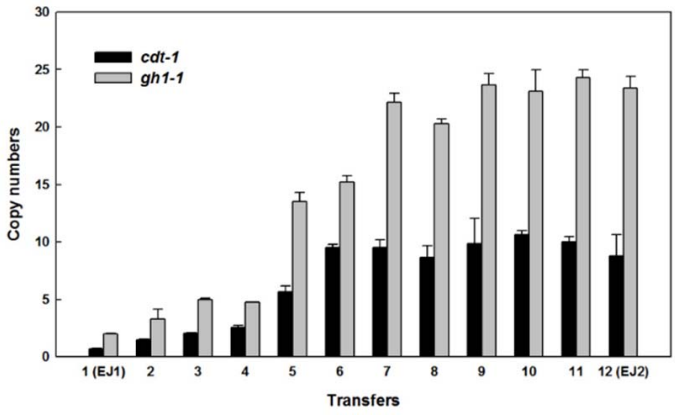
708

709

710

711

712 Figure 3.



713

714

715

716

717

718

719

720

721

722

723

724

725

726

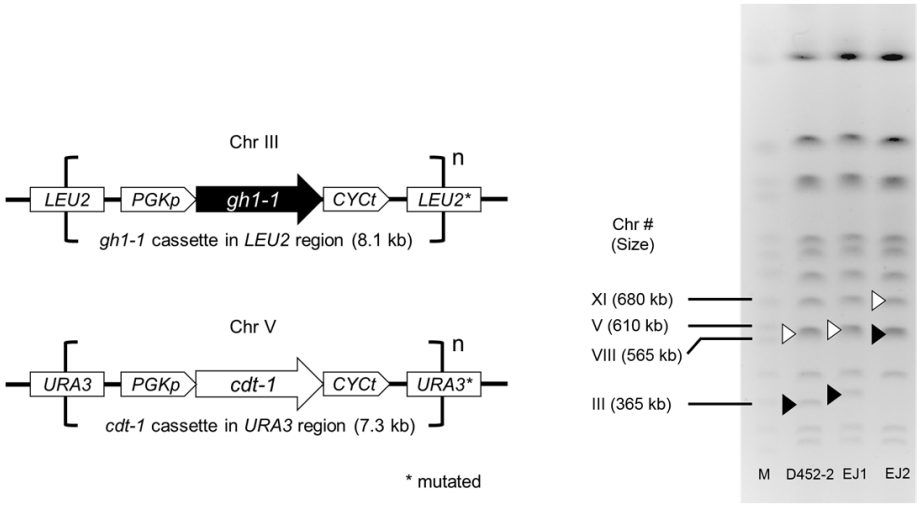
727

728

729 Figure 4.

730

731 A B



732

733

734

735

736

737

738

739

740

741

742

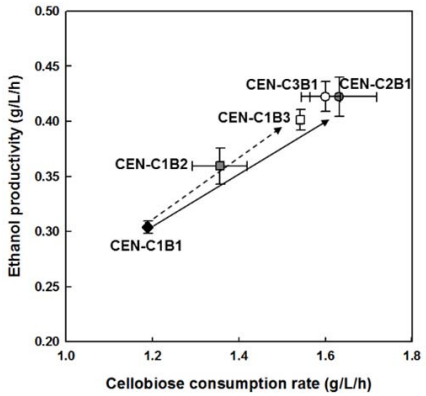
743

744

745

746 Figure 5.

747



748

749

750

751

752

753

754

755

756

757

758

759

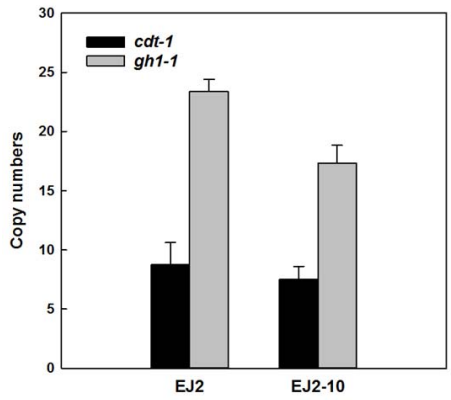
760

761

762

763 Figure 6.

764



765

766

767

768

769

770

771

772

773

774

775

776

777

778

779

780 Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
S. cerevisiae D452-2	*MATα, leu2, his3, ura3, and can1*	(43)
S. cerevisiae CEN.PK2-1D	*MATα*, *ura3-52*, *trp1-289*, *leu2-3/112*, *his3Δ1*, *MAL2-8C*, *SUC2*	(44)
S. cerevisiae EJ1	D452-2 *leu2*::*LEU2* pRS405-gh1-1 *ura3*::*URA3* pRS406-cdt-1	This study
S. cerevisiae EJ2	Evolved strain of EJ1	This study
S. cerevisiae CEN-C1B1	CEN.PK2-1D/pRS424-gh1-1/pRS426-cdt-1	This study
S. cerevisiae CEN-C1B2	CEN.PK2-1D/pRS424-gh1-1/pRS425-gh1-1/pRS426-cdt-1	This study
S. cerevisiae CEN-C1B3	CEN.PK2-1D/pRS423-gh1-1/pRS424-gh1-1/pRS425-gh1-1/pRS426-cdt-1	This study
S. cerevisiae CEN-C2B1	CEN.PK2-1D/pRS425-gh1-1/pRS424-cdt-1/pRS426-cdt-1	This study
S. cerevisiae CEN-C3B1	CEN.PK2-1D/pRS425-gh1-1/pRS423-cdt-1/pRS424-cdt-1/pRS426-cdt-1	This study
S. cerevisiae EJ1 *svl3Δ*	EJ1 *svl3*::*KanMX4*	This study
S. cerevisiae EJ1 *svl3Δ*-1S	EJ1 *svl3Δ*/pRS423-EJ1 SVL3	This study
S. cerevisiae EJ1 *svl3Δ*-2S	EJ1 *svl3Δ*/pRS423-EJ2 SVL3	This study
S. cerevisiae DCDT-1G	D452-2/pRS425-gh1-1/pRS426-cdt-1	(24)
Plasmids		
pRS405	Integration plasmid, *LEU2*	(45)
pRS406	Integration plasmid, *URA3*	(45)
pRS423	2 μm origin, *HIS3*	(46)
pRS424	2 μm origin, *TRP1*	(46)
pRS425	2 μm origin, *LEU2*	(46)
pRS426	2 μm origin, *URA3*	(46)
pRS405-gh1-1	pRS405 *PGK1p-gh1-1-CYC1t*	This study
pRS423-gh1-1	pRS423 *PGK1p-gh1-1-CYC1t*	This study
pRS424-gh1-1	pRS424 *PGK1p-gh1-1-CYC1t*	This study
pRS425-gh1-1	pRS425 *PGK1p-gh1-1-CYC1t*	(23)
pRS406-cdt-1	pRS406 *PGK1p-cdt-1-CYC1t*	This study
pRS423-cdt-1	pRS423 *PGK1p-cdt-1-CYC1t*	This study
pRS424-cdt-1	pRS424 *PGK1p-cdt-1-CYC1t*	This study
pRS426-cdt-1	pRS426 *PGK1p-cdt-1-CYC1t*	(23)
pRS423-EJ1 SVL3	pRS423 *TDH3p-SVL3* from EJ1-*CYC1t*	This study
pRS423-EJ2 SVL3	pRS423 *TDH3p-SVL3* from EJ2-*CYC1t*	This study

Table 2. Changes in cellobiose fermentation parameters[1] after serial subcultures[2]

	Value (mean ± SD)		
	EJ1	EJ2	EJ2-10
$r_{cellobiose}$	0.18 ± 0.00	2.73 ± 0.01	2.62 ± 0.10
$r_{cellobiose}^{*}$	0.09 ± 0.00	0.49 ± 0.00	0.46 ± 0.01
$Y_{ethanol}$	-	0.40 ± 0.00	0.40 ± 0.00
$P_{ethanol}$	-	1.09 ± 0.00	1.04 ± 0.03
$P_{ethanol}^{*}$	-	0.19 ± 0.00	0.18 ± 0.00

[1]Fermentation experiments were performed under oxygen-limited conditions (100 rpm) in YP medium with 80 g/L of cellobiose, and an initial cell density was adjusted to 0.29 g/L (OD_{600} = 1). The parameters were measured at 29 hours. The results are the means of duplicate experiments.

[2]Serial subcultures were performed under oxygen-limited conditions (100 rpm) in YP medium with 80 g/L of cellobiose or 20 g/L of glucose, and initial cell density of each subculture was adjusted to 0.29 g/L (OD_{600} = 1).

Parameters: $r_{cellobiose}$, cellobiose consumption rate (g/L·h); $r_{cellobiose}^{*}$, specific cellobiose consumption rate (g/g cell/h); $Y_{ethanol}$, ethanol yield (g/g cellobiose); $P_{ethanol}$, volumetric ethanol productivity (g/L·h); $P_{ethanol}$, specific ethanol productivity (g/g cell/h)