Metabolic Engineering 15 (2013) 134–143

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Energetic benefits and rapid cellobiose fermentation by *Saccharomyces cerevisiae* expressing cellobiose phosphorylase and mutant cellodextrin transporters

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ARTICLE INFO

Article history:
Received 24 August 2012
Received in revised form
8 October 2012
Accepted 5 November 2012
Available online 22 November 2012

Keywords:
Cellobiose
Phosphorylase
β-glucosidase
Cellodextrin transporter
Thermodynamics

ABSTRACT

Anaerobic bacteria assimilate cellodextrins from plant biomass by using a phosphorolytic pathway to generate glucose intermediates for growth. The yeast *Saccharomyces cerevisiae* can also be engineered to ferment cellobiose to ethanol using a cellodextrin transporter and a phosphorolytic pathway. However, strains with an intracellular cellobiose phosphorylase initially fermented cellobiose slowly relative to a strain employing an intracellular β-glucosidase. Fermentations by the phosphorolytic strains were greatly improved by using cellodextrin transporters with elevated rates of cellobiose transport. Furthermore under stress conditions, these phosphorolytic strains had higher biomass and ethanol yields compared to hydrolytic strains. These observations suggest that, although cellobiose phosphorolysis has energetic advantages, phosphorolytic strains are limited by the thermodynamics of cellobiose phosphorolysis ($\Delta G^{\circ} = +3.6$ kJ mol^{-1}). A thermodynamic "push" from the reaction immediately upstream (transport) is therefore likely to be necessary to achieve high fermentation rates and energetic benefits of phosphorolysis pathways in engineered *S. cerevisiae*.

1. Introduction

There is considerable interest in engineering microbes to convert the sugars found in plant cell walls to fuels and other chemicals (Rubin, 2008). Plant cell walls are primarily composed of cellulose (a polymer of glucose), hemicellulose (a heterogeneous polymer of pentoses, hexoses and sugar acids) and lignin (a heterogeneous phenolic polymer) (Carroll and Somerville, 2009). They are abundant in agricultural and municipal wastes, and in dedicated energy crops (Somerville et al., 2010). The yeast *Saccharomyces cerevisiae* is a favored platform for microbial engineering efforts to produce biofuels from cellulosic hydrolyzates because it is robust, simple to manipulate genetically, and capable of high carbon fluxes through central metabolic pathways (Zhang et al., 2011). Despite this *S. cerevisiae* has a number of drawbacks including an inability to naturally ferment pentose sugars (Hahn-Hagerdal et al., 2007), sensitivity to solvents (Ma and Liu, 2010), and sensitivity to inhibitory compounds found in deconstructed plant materials (Almeida et al., 2011). These drawbacks have been addressed to varying degrees (Hahn-Hagerdal et al., 2007; Nevoigt, 2008)

Another disadvantage in using *S. cerevisiae* for producing cellulosic biofuels is its inability to naturally ferment cellodextrins such as cellobiose. Cellobiose, the repeating unit of cellulose, is a β(1→4) linked disaccharide of glucose that is produced by the enzymatic digestion of cellulose by cellulases (Zhang and Lynd, 2004b). To enable cellobiose consumption, *S. cerevisiae* has been modified to either: (i) secrete or surface-display a β-glucosidase to hydrolyze cellobiose to glucose extracellularly (Machida et al., 1988); (ii) import cellobiose with a cellodextrin transporter for intracellular hydrolysis by a β-glucosidase (Galazka et al., 2010; Ha et al., 2011; Li et al., 2010); or (iii) import cellobiose with a lactose permease for intracellular phosphorolysis by a cellobiose phosphorylase (CBP) (Sadie et al., 2011). In the extracellular or intracellular hydrolytic pathways, the O-glycosidic linkage of cellobiose is cleaved by β-glucosidase (EC 3.2.1.21) with H_2O to produce glucose, while in the phosphorolytic pathway it is cleaved by cellobiose phosphorylase (EC 2.4.1.20) with inorganic phosphate (P_i) to produce glucose and α-glucose-1-phosphate (Glc-1P) (Fig. 1). The difference between the hydrolytic and

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http://dx.doi.org/10.1016/j.ymben.2012.11.005

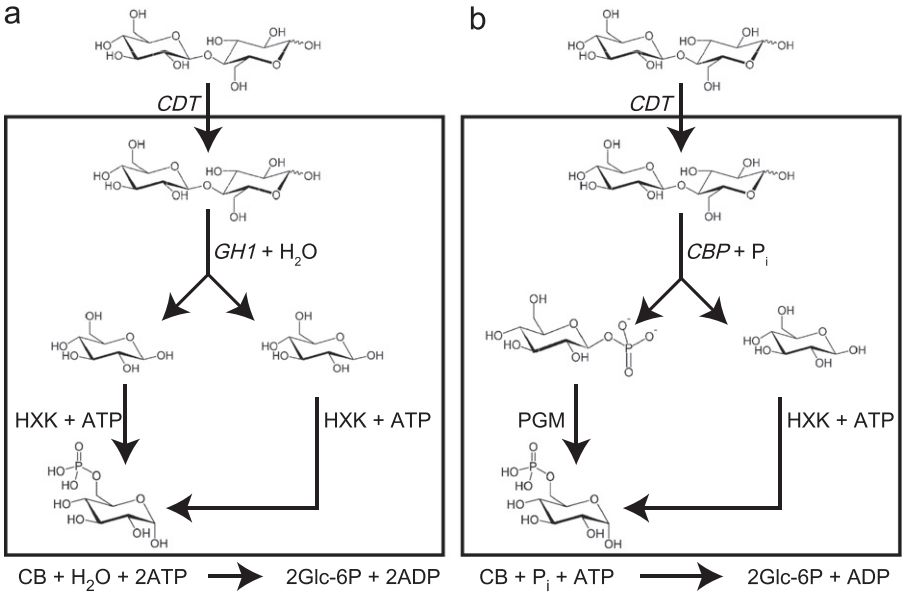

Fig. 1. Two possible cellobiose fermentation pathways. Following transport across the plasma membrane by a cellodextrin transporter (CDT), cellobiose is cleaved either (a) by hydrolysis *via* β-glucosidase (GH1 here, or GH3 (Galazka et al., 2010)) or (b) by phosphorolysis *via* cellobiose phosphorylase (CBP). Intracellular glucose is formed in the hydrolytic pathway, which is converted to glucose-6-phosphate (Glc-6P) by hexokinase (HXK). Intracellular glucose and glucose-1-phosphate (Glc-1P) is formed in the phosphorolytic pathway. Here, Glc-1P is converted to Glc-6P by phosphoglucomutase (PGM), while glucose is converted to Glc-6P by HXK. In both pathways Glc-6P is fermented to ethanol and CO_2 by endogenous yeast enzymes. Enzymes engineered into yeast are italicized.

phosphorolytic pathways is significant to cellular energetics, because the first step of the Embden-Meyerhof glycolytic pathway consumes adenosine triphosphate (ATP) to phosphorylate glucose (van Maris et al., 2006). Thus the phosphorolytic pathway may be preferable when ATP is in short supply, or under physiological conditions that demand more ATP, because less ATP is consumed for glucose phosphorylation (Fig. 1). The scarcity of ATP under anaerobic conditions may be a crucial factor in the preponderance of cellodextrin phosphorolytic pathways in obligate anaerobic bacteria (Lou et al., 1996; Zhang and Lynd, 2005).

The consequences of this difference in energetics on cellular physiology have been demonstrated with maltose fermentation (de Kok et al., 2011). Replacement of the endogenous hydrolytic pathway for maltose utilization with a heterogeneous phosphorolytic pathway in *S. cerevisiae* resulted in higher biomass yields under anaerobic conditions, presumably due to increased free energy (ATP) conservation. However, both the maltose utilization and ethanol production rates of the phosphorolytic pathway were slower than those of the hydrolytic pathway, suggesting that the energetic advantage might come with a cost of reduced bioconversion rates. We speculate that, because of the unfavorable energetics of the phosphorolysis reaction ($\Delta G^{\circ} = +3.6$ kJ mol^{-1} in the case of cellobiose phosphorolysis (Alexander, 1961), rapid substrate supply or a concentration gradient may be necessary to maintain a high flux of the reaction. Here, we compare engineered *S. cerevisiae* that rapidly ferment cellobiose to ethanol *via* intracellular cellobiose hydrolysis or phosphorolysis. We demonstrate that the phosphorolytic pathway has energetic advantages, but that rapid cellobiose fermentation rates are only realized by "pushing" the phosphorolytic reaction forward when improved cellobiose transport is facilitated by a mutant cellobiose transporter.

2. Materials and methods

2.1. Cellobiose/cellodextrin phosphorylase and phosphoglucomutase cloning

Cellobiose phosphorylase (CBP) genes from *Celvibrio gilvus* (CgCBP, Accession: AB010707), *Saccharophagus degradans* (SdCBP, Accession: YP_526792), and *Clostridium thermocellum* (CtCBP, Accession: YP_001036707), and cellodextrin phosphorylase (CDP) genes from *Clostridium lentocellum* (ClCDP, Accession: YP_00431-0865), *C. thermocellum* (CtCDP, Accession: BAA22081.1), and *Acidovibrio cellulolyticus* (AcCDP, Accession: ZP_09463103) were codon-optimized and synthesized by DNA2.0. The genes were inserted between SpeI and PstI in the 2μ plasmid, pRS425 that had been previously modified to include the *S. cerevisiae* PGK1 promoter and Cyc transcriptional terminator (PGK1_pRS425) (Galazka et al., 2010). To construct plasmids containing both a CBP gene and the *S. cerevisiae* phosphoglucomutase gene, Pgm2 (CAA89741) was first cloned between SpeI and PstI in PGK1_pRS425 to create the plasmid, PGK1_PGM_425. The Pgm2 gene bracketed by the PGK1 promoter and the Cyc transcriptional terminator was then amplified from PGK1_PGM_425. This fragment was then inserted into the SacI site of the PGK1_SdCBP_pRS425, PGK1_CgCBP_425 and PGK1_CtCBP_425 plasmids, creating the plasmids PGK1_SdCBP_PGM_425, PGK1_CgCBP_PGM_425 and PGK1_CtCBP_PGM_425. All primers are listed in Table S1.

2.2. S. cerevisiae strain construction and growth

To create the yeast strains used in this study, plasmids were transformed into the *S. cerevisiae* D452-2 (*MATα leu2 his3 ura3 can1*) (Hosaka et al., 1992) using the yeast EZ-Transformation kit (BIO 101, Vista, CA). To select transformants using an amino acid auxotrophic marker, yeast synthetic complete (YSC) medium was used, which contained 6.7 g/L yeast nitrogen base plus 20 g/L glucose, 20 g/L agar, and CSM-Leu-Trp-Ura-His (Bio 101, Vista, CA) which supplied appropriate nucleotides and amino acids.

2.3. Fermentations

A single colony from YSC plates was grown overnight in 5 mL of YP medium (10 g/L yeast extract and 20 g/L peptone) containing 20 g/L of cellobiose. Cells at mid-exponential phase were harvested and inoculated after washing twice by sterilized water. All of the flask fermentation experiments were performed using 50 mL of YP medium containing 80 or 10 g/L of cellobiose in

250 mL flask at 30 °C with initial OD_{600} of ~1.0 and under oxygen limited conditions. Acetic acid was added to deplete intracellular ATP pools (Piper, 2011). Continuous fermentations were performed using 100 mL of YP medium containing 10 g/L of cellobiose with 0.06 h^{-1} of dilution rate. Strict anaerobic cellobiose fermentations were performed using serum flask after nitrogen gas purging in minimal medium containing 6.7 g/L of yeast nitrogen base, 0.42 g/L of Tween 80, and 0.01 g/L of ergosterol. Cell growth was monitored by optical density (OD) at 600 nm using a UV-visible Spectrophotometer (Biomate 5, Thermo, NY) and OD was converted to dry cell weight (DCW) using the following conversion factor: DCW (g/L)=OD × 0.3. Cellodextrin fermentations were performed in YP medium containing 5 g/L of cellobiose, cellotriose or cellotetraose. A 96 well plate was used to monitor cell growth on cellodextrin as a sole carbon source. A culture volume in each well was 200 μL and 50 μL of mineral oil was overlaid on the top of the culture to prevent evaporation of the medium during growth measurement. The initial cell density was adjusted to OD600=~0.2. A Synergy H4 hybrid Microplate Reader (BioTek Instruments Inc., Winooski, VT) was used for measuring absorbance at 600 nm with a continuous mixing option. Glucose, 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.

2.4. Directed evolution of phosphorolytic strains

An enrichment culture was started as described in the main text using the *S. cerevisiae* D452-2 transformed with *cdt*-1 and the *S. degradans* CBP. When cellobiose concentrations dropped to almost zero, cells were collected and used to establish new cultures at an OD (600 nm) of ~0.01. This process was repeated seven times over the course of 30 days. At this point, cells were plated onto YSC plates containing 20 g/L of cellobiose to isolate fast-growing clones. After confirming the improved phenotype of isolated clones, plasmids were isolated from one of representative clone and sequenced.

2.5. Site directed mutagenesis and transporter kinetics

Site directed mutagenesis was performed using the Quikchange® protocol (Zheng et al., 2004). The primers used to introduce each mutation are listed in Table S2. Transporter kinetics were measured using the oil-stop method as described previously with slight modifications (Arendt et al., 2007; Galazka et al., 2010). Briefly, D452-2 transformed with mutant transporters inoculated at an OD (600 nm) of ~0.2 into 50 mL of DOB—uracil and grown to an OD (600 nm) of ~1. Cells were then harvested by centrifugation, washed 3× with 10 mL of transport buffer (30 mM MES-NaOH [pH 5.6], 50 mM EtOH), and resuspended to a final OD (600 nm) of ~40 in transport buffer. The GFP fluorescence of 100 μL of these cells was determined with excitation/emission wavelengths of 485/535 nm Beckman Coulter Paradigm™ plate reader. All strains had approximately equivalent levels of fluorescence (±20% of WT). To record linear rates of uptake over the course of 95 s, 50 μL of cells were added to 50 μL of [^{3}H]-cellobiose at the appropriate concentration with a final specific activity of 40 μCi/μmol in transport buffer layered over 100 μL of silicone oil (Sigma 85419). Reactions were stopped by spinning cells through the oil for 1 min at 17,000g, tubes were frozen in ethanol/dry ice, and tube-bottoms containing the cell-pellets were clipped off into 1 mL of 0.5 M NaOH. The pellets were solubilized overnight, 5 mL of Ultima Gold scintillation fluid added, and CPM determined in a Tri-Carb 2900TR scintillation counter. [^{3}H]-cellobiose was purchased from Moravek Biochemicals, Inc., and had a specific activity of 4 Ci/mmol and a purity of >99%. V_{max} and K_M values were determined by fitting a single rectangular, 2-parameter hyperbolic function to a plot of rates vs. cellobiose concentration by non-linear regression in SigmaPlot®.

2.6. Measurement of enzymatic activity in cell extracts

Fermentation reactions were inoculated as described above in either YPC80 or YPD80 (10 g/L yeast extract, 20 g/L peptone, 80 g/L glucose). At an OD (600 nm) of ~10, which corresponds to the exponential phase of the reaction, 2 mL of the culture was removed and cells pelleted. The cell pellet was washed 2× in 500 μL of ice-cold extraction buffer (50 mM HEPES-NaOH [pH 6.0], 2 mM DTT, and Roche Complete EDTA-free Protease Inhibitor Cocktail), and resuspended in 200 μL of this buffer and transferred to a screw-cap tube containing ~100 μL of 0.4 mm Zirconia/Silica beads. Cells were then lysed by bead-beating 3× for 30 s at 4 °C using a Biospec Products Mini-BEADBEATER with a 30 s pause between runs. Debris was then pelleted, and the concentration of protein in the supernatant determined by the Bradford assay (Bradford, 1976) using reagents and the microtiter plate protocol from Bio-Rad.

The amount of "cellobiase activity" in cell extracts was determined by the Glucose oxidase/Peroxidase assay (Venardos et al., 1980). 10 μg of cell extract was added to 1 mL of an assay mixture consisting of 50 mM phosphate buffer [pH 6.0], 10 U glucose oxidase, 10 U peroxidase, 1 mM o-dianisidine, and 10 mM cellobiose. The number of pmol of glucose produced per second was calculated by multiplying the rate of increase at 436 nm by 1.17×10^3, a conversion that was established from a glucose standard curve.

The amount of hexokinase activity in cell extracts was determined by coupling the production of glucose-6-phosphate to the reduction of $NADP^+$ by glucose-6-phosphate dehydrogenase (Bergmeyer et al., 1983). An amount of 10 μg of cell extract was added to 1 mL of an assay mixture consisting of 50 mM Tris–HCl [pH 8.0 at 30 °C], 13.3 mM $MgCl_2$, 540 μM ATP, 20 μM $NADP^+$, 1 U *S. cerevisiae* glucose-6-phosphate dehydrogenase, and 112 mM glucose. The number of pmol of glucose-6-phosphate produced per second was calculated by multiplying the rate of increase at 340 nm by 1.85×10^3, a conversion that was established from a glucose-6-phosphate standard curve.

2.7. Transglycosylation activity of GH1-1 and SdCBP

To measure the transglycosylation activity of GH1-1 and SdCBP, 100 nkat of each enzyme was incubated at 37 °C with 100 μL 20% (w/v) cellobiose in 50 mM phosphate buffer [pH 6.0] and 2 mM DTT. After 12 h, reactions were quenched in 400 μL 0.1 M NaOH and analyzed by ion chromatography with a Dionex ICS-3000, using a CarboPac PA200 column. Peaks were detected with an electrochemical detector.

2.8. Kinetic parameters of GH1-1 and SdCBP

The kinetic parameters of purified GH1-1 and SdCBP were determined by measuring the rate of glucose production at a variety of cellobiose concentrations using the glucose oxidase/peroxidase assay in a manner identical to that used on cell extracts detailed above. A 1 mL assay included either 8.75 pmol of GH1-1 or 20 pmol SdCBP. V_{max} and K_M values were determined by fitting a single rectangular, 2-parameter hyperbolic function to a plot of glucose production rates vs. cellobiose concentration by non-linear regression in SigmaPlot®.

3. Results

3.1. Assimilation of cellodextrin through the phosphorolytic pathway in engineered yeast

Cellobiose phosphorylase genes from *Saccharophagus degradans* (SdCBP), *C. gilvus* (CgCBP), and *C. thermocellum* (CtCBP) were codon-optimized, synthesized and cloned into multi-copy plasmids under the control of a constitutive promoter (P_{PGK}). These plasmids were transformed, along with a multi-copy plasmid carrying a cellodextrin transporter gene (*N. crassa* CDT-1) tagged with green fluorescent protein (GFP) into *S. cerevisiae* strain D452-2. All three resulting strains fermented cellobiose into ethanol (Fig. 2a, Supplementary Fig. S1). The cellobiose consumption rates of the strains expressing CgCBP, SdCBP, or CtCBP were similar (0.61–0.69 g cellobiose/L h), as were the ethanol production rates of these strains (0.22–0.26 g ethanol/L h). Cellobiose fermentation rates by the engineered strains with the phosphorolytic pathway were much slower than those by the engineered strains with the hydrolytic pathway (Ha et al., 2011). After 108 h, almost all cellobiose was fermented to ethanol with yields of 0.37–0.38 g ethanol/g cellobiose, and the amount of accumulated acetate, glucose or glycerol was less than 1 g/L. Previously developed cellobiose-transporting strains that rely on intracellular cellobiose hydrolysis ferment cellobiose with a concomitant build-up of glucose and cellodextrins (cellotriose and cellotetraose) in the extracellular media (Ha et al., 2011). However, this phenomenon was not observed with the phosphorolytic strains reported here.

CBPs have reduced activity on longer cellodextrins such as cellotriose and cellotetraose (Reichenbecher et al., 1997) that are produced by cellulases. Therefore, we also constructed strains with codon-optimized cellodextrin phosphorylases (CDP) from *C. lentocellum* (ClCDP), *C. thermocellum* (CtCDP), or *A. cellulolyticus* (AcCDP). Only ClCDP showed slight cell growth on cellodextrins when overexpressed in the presence of wild type (WT) CDT-1 (Fig. 2b).

CBP phosphorolytically cleaves cellobiose to glucose and α-glucose-1-phosphate (Glc-1P). Glc-1P must be converted to glucose-6-phosphate (Glc-6P) by phosphoglucomutase (EC 5.4.2.2) to enter the glycolytic pathway. In *S. cerevisiae*, *PGM2* is transcriptionally down-regulated during glycolytic growth (Oh and Hopper, 1990). Therefore strains in which *PGM2* was overexpressed ectopically along with *cdt*-1 and a CBP were tested. However, these *PGM2*-overexpressing strains showed slightly reduced performance as compared to those without ectopic *PGM2* (Fig. 2c, Supplementary Fig. S1).

3.2. A mutant cellobiose transporter facilitating faster cellobiose fermentation in yeast

As cellobiose fermentation rates through the phosphorolytic pathway were slower than those through the hydrolytic pathway, an evolutionary engineering approach was undertaken to improve the strain with CDT-1 and SdCBP. Fast growing mutants of the strain were enriched by serial transfer into growth media containing 80 g/L cellobiose as a carbon source. An improved strain emerged that consumed cellobiose and produced ethanol approximately 3-fold faster than the parental strain, a rate comparable to the hydrolytic pathway (Fig. 3a,b). To identify mutations responsible for this improvement, both multi-copy plasmids (pRS425-SdCBP and pRS426-CDT-1-GFP) isolated from the evolved strain were sequenced. Sequence analysis of the plasmids identified a single nucleotide mutation (C639A) in the *cdt-1* open reading frame, corresponding to a change of phenylalanine to leucine at position 213 (F213L) in the translated polypeptide. This single mutation explains the improved performance, as retransforming the isolated plasmid into un-evolved *S. cerevisiae* recapitulated the faster fermentation phenotype (Fig. 3b,c). Strains with CDT-1 (F213L) and SdCBP consumed cellobiose and produced ethanol with rates of 1.72 ± 0.11 g cellobiose/L h and 0.74 ± 0.05 g ethanol/L h (0.25 ± 0.02 g ethanol/g cell h), respectively. This is an improvement of 249% and 290% in the rate of cellobiose consumption, and ethanol production, respectively. The ethanol yield was improved from 0.37 g/g to 0.44 g/g, and the amount of accumulated acetate, glucose, glycerol, or cellodextrins was less than 1 g/L. Notably, a strain with the mutant CDT-1 (F213L) and ClCDP also exhibited much faster cell growth on cellodextrins compared to a strain with WT CDT-1 (Fig. 3d). Additionally, strains with CDT-1 (F213L) and either CgCBP or CtCBP also grew two to three times faster than equivalent strains with WT CDT-1 (Supplementary Fig. S1e and S1f).

Strains with CDT-1 and an intracellular β-glucosidase (GH1-1) were previously shown to ferment cellobiose to ethanol (Galazka et al., 2010; Ha et al., 2011; Li et al., 2010). To assess whether the F213L mutant of CDT-1 would also improve the performance of this hydrolytic strain, *cdt-1* (F213L) was transformed into

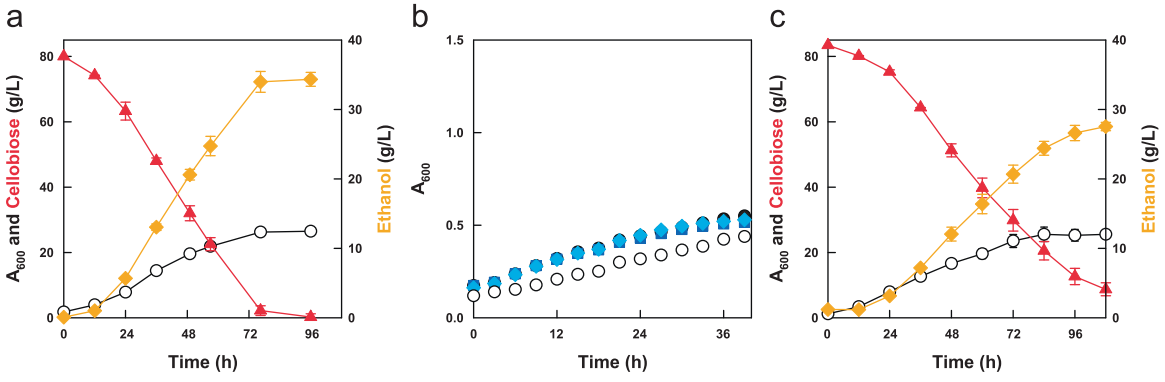

Fig. 2. Cellobiose fermentation profiles of engineered yeast strains with CDT-1 and cellobiose and cellodextrin phosphorylases. *Saccharomyces cerevisiae* D452-2 was transformed with a plasmid containing *cdt*-1, and a second plasmid containing a codon-optimized cellobiose phosphorylase from *S. degradans* under the control of the same promoter and terminator. (a) The cellobiose fermentation profile of this strain under oxygen limited conditions is shown; cellobiose (▲), ethanol (♦), and biomass (○). (b) *S. cerevisiae* was transformed with a plasmid encoding the WT cellodextrin transporter, CDT-1, and a second plasmid with the cellodextrin phosphorylase from *Clostridium lentocellum*, ClCDP. Growth curves of cellodextrin fermentation of these strains using a 96 well plate are shown; cellobiose (●), cellotriose (■), or cellotetraose (♦) as substrate. The parental strain (*S. cerevisiae* D452-2) was used as a negative control (○). (c) The cellobiose fermentation profile as in (a), of *S. cerevisiae* D452-2 harboring an additional plasmid containing *PGM2*. In both panels, values are the mean of two independent fermentations, and error bars represent the standard deviation between these.

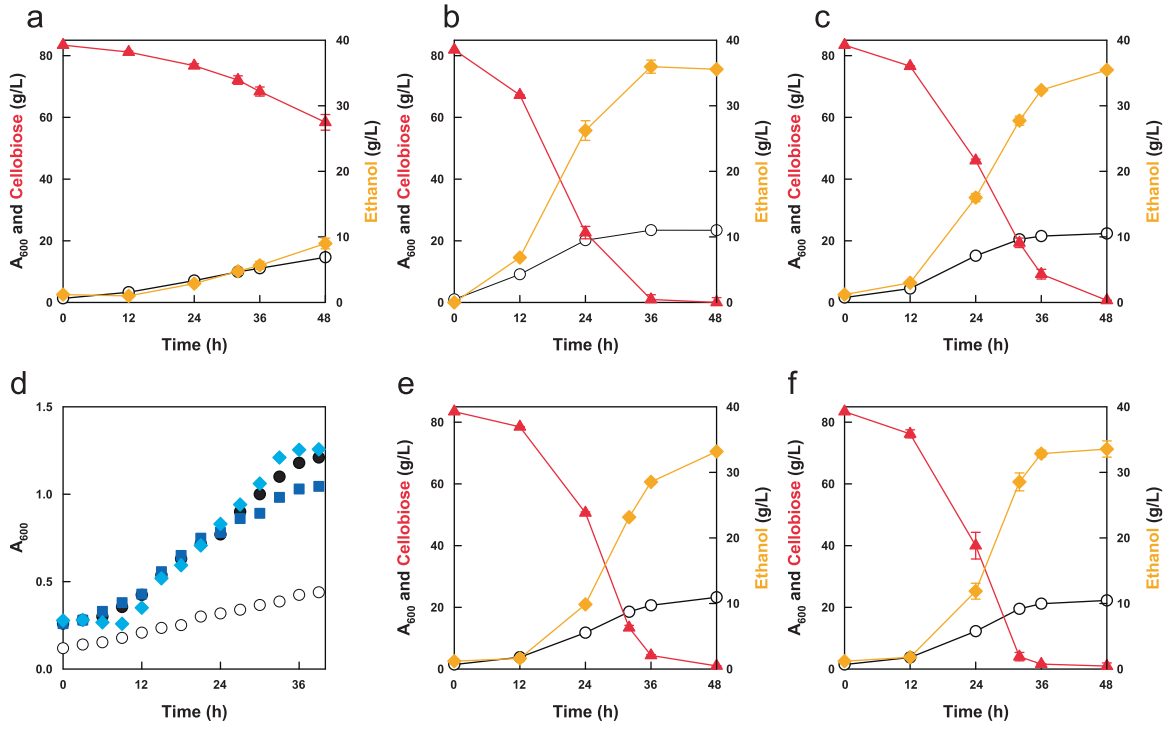

Fig. 3. Fermentation profiles of yeast harboring wild type CDT-1 or a spontaneously derived mutant of CDT-1. (a) *S. cerevisiae* was transformed with a plasmid encoding the WT cellodextrin transporter, CDT-1 and a second plasmid with the cellobiose phosphorylase, SdCBP. (b) An evolved *S. cerevisiae* transformed with a plasmid encoding CDT-1 and a second plasmid with SdCBP was isolated after evolutionary engineering approach. (c) *S. cerevisiae* was transformed with an evolved mutant of cellodextrin transporter, CDT-1 (F213L) and a second plasmid with the cellobiose phosphorylase, SdCBP. The cellobiose fermentation profiles of these strains under oxygen limited conditions are shown; cellobiose (▲), ethanol (◆), and biomass (○). (d) *S. cerevisiae* was transformed with a plasmid encoding an evolved mutant of this transporter, CDT-1 (F213L), and a second plasmid with the cellodextrin phosphorylase, ClCDP. Growth curves of cellodextrin fermentation of these strains using 96 well plate are shown; cellobiose (●), cellotriose (■), or cellotetraose (◆) as substrate. The parental strain (*S. cerevisiae* D452-2) was used as a negative control (○). (e and f) *S. cerevisiae* was transformed with one plasmid containing either the WT cellodextrin transporter, *cdt-1*, (e) or an evolved mutant of this transporter, *cdt-1* (F213L), (f) and a second plasmid with the β-glucosidase, *gh1-1*. Symbols are the same as in (a). In all panels, values are the mean of two independent fermentations, and error bars represent the standard deviation between these.

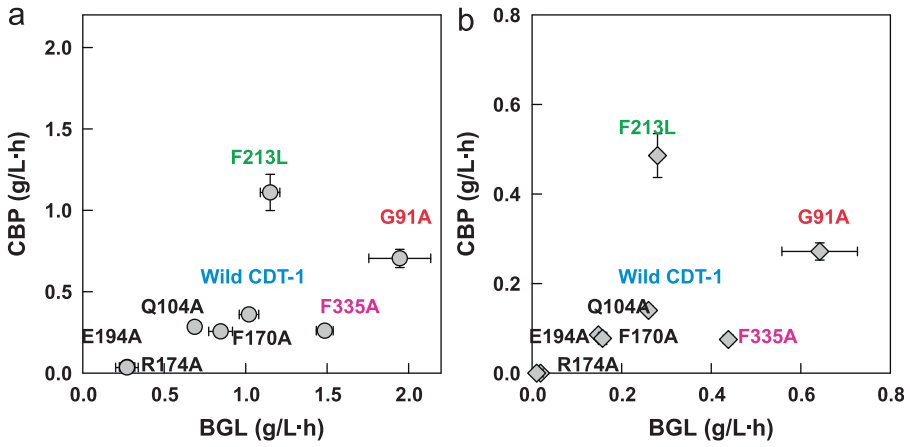

Fig. 4. Cellobiose consumption and ethanol production rates by engineered strains with various CDT-1 mutants with either phosphorolytic or hydrolytic pathways. The D452-2 strain of *S. cerevisiae* was transformed with a plasmid containing either WT *cdt-1* or a *cdt-1* mutant, and a second plasmid containing codon-optimized cellobiose phosphorylase from *S. degradans* or the β-glucosidase, *gh1-1*. The rate at which these strains consumed cellobiose (a) and produced ethanol (b) is shown. Ethanol productivities with various *cdt*-1 mutants and cellobiose phosphorylase are plotted along the y-axis, while productivities with various *cdt-1* mutants and β-glucosidase are plotted along the *x*-axis. All values are the mean of two independent fermentations, and error bars represent the standard deviations between these.

S. cerevisiae along with the intracellular β-glucosidase (*gh1-1*). Only a slight improvement in the cellobiose fermentation parameters was seen compared to a strain with WT CDT-1 and GH1-1 (Fig. 3e,f). As previously reported (Ha et al., 2011), cellobiose fermentation by strains with CDT-1 and GH1-1 occurs with an extracellular build-up of glucose and cellodextrins, and the same pattern was seen in strains with CDT-1 (F213L).

3.3. Investigation of metabolic control of cellobiose utilization by various cellobiose transporter mutants

To determine if other mutants of CDT-1 would have similar effects on cellobiose fermentation, ninety six CDT-1 mutants were constructed which have replacements of one conserved amino acid to alanine. They were introduced into *S. cerevisiae* along with

*gh*1-1 and their growth rates with cellobiose as a carbon source were measured briefly using 96 well plates (data not shown). On the basis of growth rates, we selected two fast mutants (G91A and F335A), two moderate mutants (Q104A and F170A), and two slow mutants (E194A and R174A). Cellobiose consumption and fermentation rates of strains with these transporters and GH1-1 followed a different trend than that observed in strains expressing one mutant transporter and SdCBP (Fig. 4, Supplementary Fig. S2). For example, the optimal mutant transporter was different for strains with GH1-1 than for strains with SdCBP.

To assess transporter function directly, the kinetic properties of the four transporters giving the best fermentation rates in the respective hydrolytic and phosphorolytic pathways (WT, G91A, F335A, F213L) were determined by measuring the rate of [^{3}H]-cellobiose uptake into cells (Table 1 and Supplementary Fig. S3). All mutant transporters had reduced affinity for cellobiose (higher K_M). However, the fermentation conditions were carried out in cellobiose concentrations far above the measured K_M values for the transporters, indicating that V_{max} values for these transporters affect the fermentation parameters. Compared to WT, all of the transporter mutants had higher maximal cellobiose transport rates (V_{max}).

In the above measurements of transporter kinetics, strains were grown on glucose, which resulted in each transporter being expressed at similar levels ($\pm\sim$20% of WT) as judged by the amount of GFP fluorescence per cell when cell densities were adjusted to an OD of $\sim$10. However, during fermentation of cellobiose, there were large differences in the amount of GFP fluorescence per cell depending upon which transporter mutant was being expressed (Supplementary Fig. S4). Furthermore, these differences were strongly correlated with cellobiose consumption and ethanol production rates. These results suggest that better expression or localization of mutant cellodextrin transporters is important for efficient cellobiose fermentation, regardless of whether the hydrolytic or phosphorolytic pathway is used. Furthermore, transporters that transport cellobiose at a higher maximum velocity allow for faster fermentation rates.

3.4. Comparison of catalytic properties of β-glucosidase and cellobiose phosphorylase

The distinct differences in strain performance when cellobiose is cleaved by hydrolysis versus phosphorolysis despite the use of identical transporter genes, suggests that the fate of transported cellobiose depends upon the mechanism and kinetics of its intracellular processing. Therefore the intracellular metabolism of these strains was analyzed by measuring select enzymatic activities in whole cell extracts. To determine if there was a significant difference in the ability of the two strains to cleave intracellular cellobiose, the amount of "cellobiase activity" in 10 μg of cellular extract (as determined by the Bradford assay) was measured (Supplementary Fig. S5). "Cellobiase activity", defined here as the rate of glucose produced from saturating cellobiose regardless of the mechanism, was similar in strains with GH1-1 or SdCBP. This assay underestimates the activity of CBP by 50% because the Glc-1P produced by this enzyme is not accounted for, suggesting that extracts from SdCBP cultures had about two-fold higher maximal activity for the cleavage of cellobiose compared to the hydrolytic strains. The amount of hexokinase activity in 10 μg of these extracts was indistinguishable between strains with GH1-1 and SdCBP, with both of these strains having slightly more hexokinase activity than WT D452-2 grown on glucose (Supplementary Fig. S5). We also determined kinetic parameters of GH1-1 and SdCBP after purification. While both enzymes showed similar k_{cat} ($\sim$7 s^{-1}), GH1-1 showed lower K_M (0.32 mM) than SdCBP (0.65 mM) for cellobiose (Fig. 5a,b). This could explain why we observed faster cellobiose fermentation with SdCBP with the CDT-1 (F213L) mutant transporter exhibiting improved cellobiose transport, as higher intracellular cellobiose concentrations would be required for SdCBP to work with maximum efficiency.

As noted, during cellobiose fermentations performed with strains with CDT-1 variants and GH1-1, glucose and cellodextrins accumulated in the media. Yet this is not observed during fermentations with strains bearing CDT-1 and SdCBP. The accumulation of glucose and cellodextrins is presumably due to their efflux following either cellobiose hydrolysis or transglycosylation. Transglycosylation by β-glucosidases is a well-documented activity (Saloheimo et al., 2002) in which high concentrations of glucose and cellobiose can be dehydrated to cellodextrins. To assay the transglycosylation activity of GH1-1 and SdCBP, both enzymes were enriched from cellular extracts by affinity chromatography (Methods). The enriched enzymes were incubated with 20% (w/v) cellobiose for 24 h, and the reaction products were analyzed by HPLC. GH1-1 but not SdCBP had significant transglycosylation activity under these conditions, producing both cellotriose and cellotetraose from cellobiose (Fig. 5c).

3.5. Energetic benefits of phosphorolysis over hydrolysis under anaerobic and stress conditions

Phosphorolysis should allow *S. cerevisiae* to capture more energy from cellobiose. To measure any energetic advantages of cellobiose phosphorolysis compared to hydrolysis, four *S. cerevisiae* strains were constructed that had either the hydrolytic pathway coupled with either WT CDT-1 or mutant CDT-1 (F213L) (C_WH and C_MH, respectively) or the phosphorolytic pathway coupled with either WT CDT-1 or mutant CDT-1 (F213L) (C_WP and C_MP, respectively) (Fig. 6). First, we looked for differences under strict anaerobic conditions when ATP generation is limited. When the WT CDT-1 was used, the cellobiose consumption rate of the phosphorolytic strain (C_WP) was much slower than that of the hydrolytic strain (C_WH) (Fig. 6a and Supplementary Fig. S6). This matched the results in aerobic conditions (Fig. 3a,e). However, with the mutant CDT-1 (F213L), the phosphorolytic strain (C_MP) exhibited higher (44%) cell growth than the hydrolytic strain (C_MH) with mutant CDT-1 (F213L), corresponding to higher (32–35%) cellobiose consumption and ethanol production (Fig. 6a and Supplementary Fig. S6). We next looked for differences under continuous culture conditions. At steady-state, biomass yields by the phosphorolytic strain were significantly higher (28–35%) than those by the hydrolytic strain while ethanol yields were similar (Fig. 6b and Supplementary Fig. S7). The behavior of these strains during the fermentation of 10 g/L cellobiose was then determined in complex (YP) or minimal (SC) media with and without acetic acid. Acetic acid depletes the ATP pool in yeast and its presence should enhance any differences in the ATP yield of the two pathways (Piper, 2011). The phosphorolytic strain with WT CDT-1 (C_WP) consistently exhibited higher

Table 1
Transport kinetics of WT CDT-1 and CDT-1 mutants.

	K_M (mM)	V_{max} (pmol/s, normalized to 2×10^8 cells)	V_{max} (pmol/s, normalized to GFP fluorescence)
WT	0.0076 ± 0.0015	0.60 ± 0.03	0.86 ± 0.05
F213L	0.1888 ± 0.0553	2.66 ± 0.37	2.38 ± 0.31
F335A	0.1148 ± 0.0464	1.64 ± 0.24	1.75 ± 0.26
G91A	0.0435 ± 0.0101	1.51 ± 0.10	1.87 ± 0.12

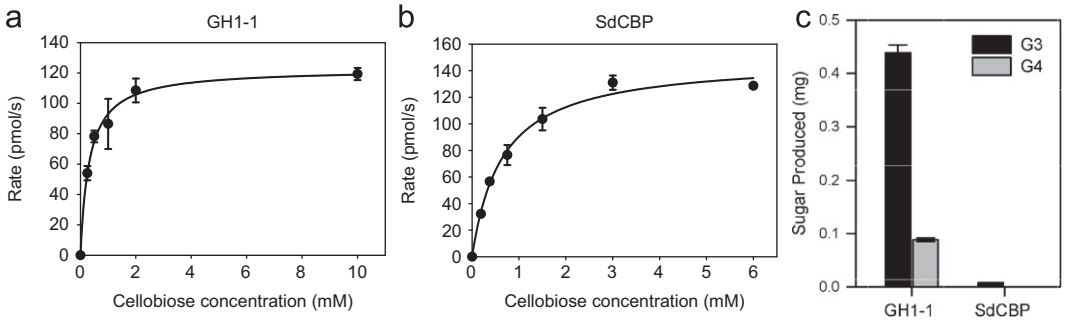

Fig. 5. Catalytic properties of β-glucosidase and cellobiose phosphorylase. GH1-1 (a) and the *S. degradans* cellobiose phosphorylase (SdCBP) (b) were purified directly from the strains being studied. Linear rates of catalysis were determined at a variety of cellobiose concentrations in triplicate, and kinetic parameters determined by non-linear regression. (c) Transglycosylation activity of GH1-1 and *S. degradans* cellobiose phosphorylase. 200 pkat of either purified GH1-1 or SdCBP was incubated with 20% (w/v) cellobiose for 24 h at 30 °C in 50 mM phosphate buffer at pH 6.0. The same incubation was carried out without the addition of enzyme as a control. The products were then analyzed by HPLC.

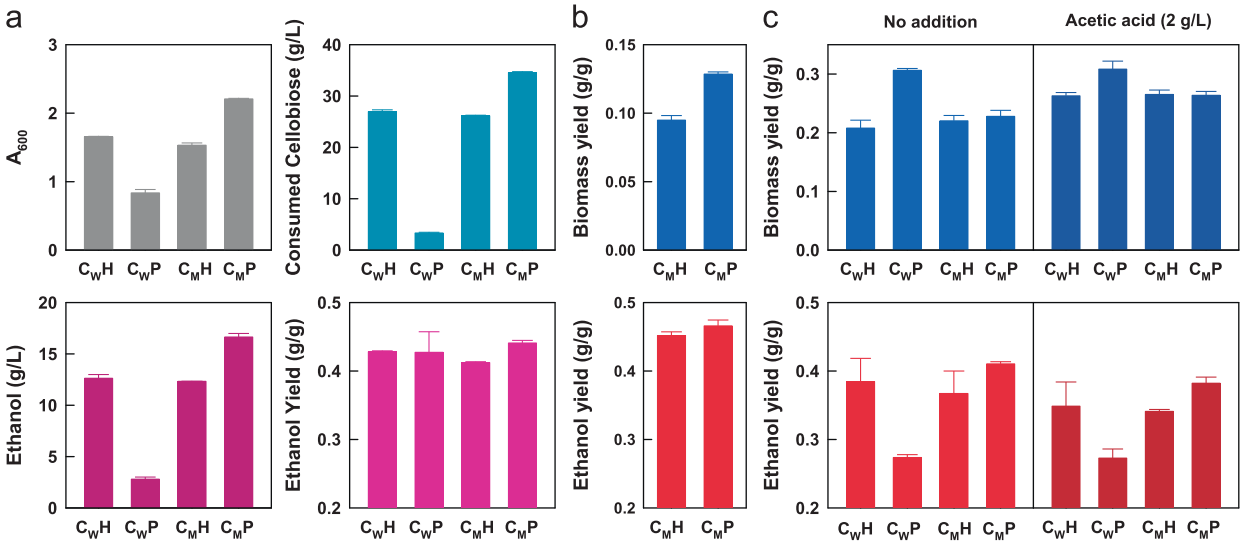

Fig. 6. Physiological characterization of the cellobiose hydrolytic or phosphorolytic strain. To evaluate energetic advantages of cellobiose phosphorolysis as compared to hydrolysis, cellobiose fermentation experiments were performed under various conditions. (a) Strict anaerobic fermentations were performed in YSC medium containing 10 g/L of cellobiose by engineered *S. cerevisiae* strains transformed with a plasmid containing either *gh1-1* (H) or SdCBP (P) and a second plasmid with either wild type *cdt-1* (C_W) or mutant *cdt-1* (F213L) (C_M), yielding the four strains, C_WH, C_WP, C_MH, and C_MP. (b) Continuous fermentations were performed using YP medium containing 10 g/L of cellobiose by engineered *S. cerevisiae* (C_MH and C_MP) with 0.06 h^{-1} dilution rate. (c) Flask fermentations were performed in YP medium containing 10 g/L of cellobiose with or without acetic acid (2 g/L) addition by engineered *S. cerevisiae* strains (C_WH, C_WP, C_MH, and C_MP).

biomass yields under all tested conditions as compared to the analogous hydrolytic strain (C_WH). However, it consumed cellobiose slowly and produced less ethanol (Fig. 6c and Supplementary Fig. S8). The phosphorolytic strain with the F213L mutant of CDT-1 (C_MP) showed similar biomass yields as compared to the analogous hydrolytic strain (C_MH), but resulted in higher ethanol yields under all tested conditions (Fig. 6c and Supplementary Fig. S8). Similar phenomena were observed when minimal media (SC) was used instead of complex media (YP) (Supplementary Fig. S9). In summary, we were able to observe consistent energetic benefits (better cell growth and improved resistance to stress conditions) to using the phosphorolytic pathway.

4. Discussion

Here we describe *S. cerevisiae* strains engineered to rapidly ferment cellobiose *via* a phosphorolytic pathway. We accomplished this by introducing two genes into *S. cerevisiae*: a mutant cellodextrin transporter and an intracellular cellobiose phosphorylase. A similar strain was first reported by Sadie et al. (2011).

The Sadie et al. strain consumed ~10 g/L of cellobiose in ~114 h, but was not shown to produce ethanol or other fuels and chemicals. In contrast, the best strain reported here consumed 80 g/L cellobiose in ~36 h, producing ethanol with a yield of ~0.44 g ethanol/g cellobiose. This rate matches that of previously described strains that used the cellodextrin transporter, but instead contained an intracellular β-glucosidase (Ha et al., 2011). Notably, cellobiose phosphorolysis leads to consistently higher ethanol yields than the hydrolytic pathway. We speculate that the improved yields are in part due to a lack of cellodextrin accumulation during fermentations performed with phosphorolytic strains. In contrast, when engineered strains with the hydrolytic pathway are used to ferment cellobiose, cellodextrins accumulate in the media. Hydrolytic strains can consume the accumulated cellodextrins after depletion of cellobiose, but the rate and yield of cellodextrin fermentation is lower than that of cellobiose fermentation. As a result, engineered strains with the hydrolytic pathway exhibited a biphasic fermentation pattern: rapid early-stage

cellobiose fermentation, followed by slow late-stage cellodextrin fermentation. In contrast, engineered strains with the phosphorolytic pathway showed a consistent cellobiose fermentation rate, without cellodextrin accumulation. This monophasic fermentation pattern was observed regardless of the initial cell density. As a result, the ethanol productivity of this strain increased linearly with increasing initial cell densities, up to 1.90 g ethanol/L h (0.23 g ethanol/g cell h) with an initial OD (600 nm) of 21.1 which is equivalent to ~6 g cell/L (Supplementary Figs. S10 and S11).

In the present study, energetic advantages were consistently observed when cellobiose was fermented through the phosphorolytic pathway. In theory once cellobiose is transported into the cell, only 1 ATP is consumed for every 2 Glc-6P produced through the phosphorolytic pathway, while 2 ATP are consumed through the hydrolytic pathway. This should increase the ATP yield from the fermentative metabolism of 1 intracellular cellobiose to 5 ATP in phosphorolytic strains. By contrast, hydrolytic strains should yield 4 ATP per fermented cellobiose. Increased cellular ATP can lead to higher biomass yields per gram of carbon, an effect that we observed under various conditions. The same effect was documented by de Kok et al. (2011), who engineered a phosphorolytic maltose consumption pathway into *S. cerevisiae* by replacing the native maltases with a maltose phosphorylase. They observed 26% more biomass production in phosphorolytic strains grown on maltose. Similarly, we observed higher biomass yields (28–35%) under steady state conditions and enhanced cell growth, cellobiose consumption, and ethanol production (32–44%) under strict anaerobic conditions when cellobiose was consumed by phosphorolysis.

Pathways with high ATP yields may be particularly important during the production of fuels and chemicals from lignocellulose for at least two reasons. First, fermentations of lignocellulosic sugars are usually performed in oxygen-limited or anaerobic conditions when respiration is impossible and ATP yields are thus constrained. Second, lignocellulose is commonly treated with dilute acid to hydrolyze hemicellulose and liberate cellulose from lignin (Humbird, 2011). Subsequent enzymatic hydrolysis of cellulose results in a low pH hydrolyzate containing not only hexose and pentose sugars, but also high concentrations of hemicellulose-derived acetic acid (Almeida et al., 2011). At low pH, acetic acid moves freely across the plasma membrane of *S. cerevisiae* and into the cytosol, where it deprotonates into acetate (Casey et al., 2010). To maintain homeostasis, the dissociated proton and acetate must be exported through the membrane-bound H^+ pump, Pma1p (Russell, 1992), and the weak acid efflux pump, Pdr12p (Piper et al., 1998), both of which consume ATP directly or indirectly. For both of these reasons, the energetic benefits of the phosphorolytic pathway may be particularly important for microbial plant cell wall conversion processes.

The improved ATP yields of the phosphorolytic pathway are only possible because the phosphorolytic pathway dissipates less free energy in the early steps of glycolysis (Supplementary Fig. S12). The phosphorolytic pathway begins with the phosphorolysis of cellobiose to glucose and Glc-1P ($\Delta G^\circ = 3.6$ kJ/mol) (Alexander, 1961). This Glc-1P is converted to Glc-6P by phosphoglucomutase ($\Delta G^\circ = -7.0$ kJ/mol) (Goldberg and Tewari, 1989), while glucose is phosphorylated to Glc-6P by hexokinase ($\Delta G^\circ = -16.7$ kJ/mol) (Goldberg, 1975) in a reaction that consumes 1 ATP. Thus, the overall free energy change associated with the phosphorolytic conversion of intracellular cellobiose to 2 Glc-6P is approximately −20.1 kJ/mol, with 1 ATP consumed in the process. In contrast, the hydrolytic pathway begins with the hydrolysis of cellobiose to 2 glucose ($\Delta G^\circ = -12.5$ kJ/mol (Tewari and Goldberg, 1989)), followed by the phosphorylation of each glucose by hexokinase ($\Delta G^\circ = -33.4$ kJ/mol). Thus, the overall free energy change associate with the hydrolytic conversion of intracellular cellobiose to 2 Glc-6P is −45.9 kJ/mol, with 2 ATP consumed in the process.

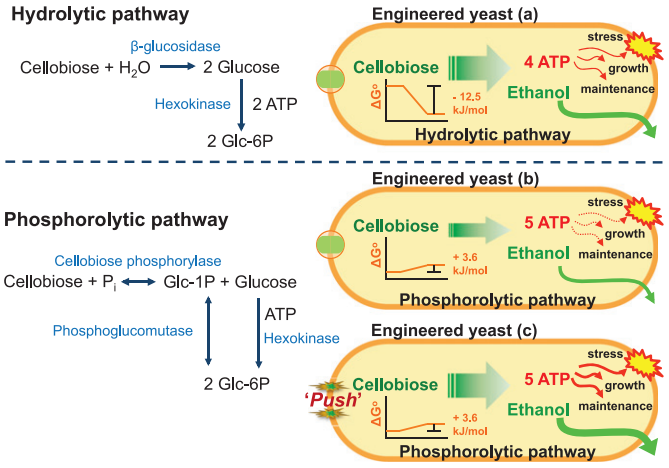

Fig. 7. Cellobiose fermentation pathways in yeast require a thermodynamic "push." Following transport across the plasma membrane by a cellodextrin transporter (CDT), cellobiose is cleaved either by hydrolytic (a) or phosphorolytic pathways (b, c) in the engineered yeast. The hydrolytic pathway consumes cellobiose faster than the phosphorolytic pathway because of the negative Gibbs free energy (ΔG°) of cellobiose hydrolysis, although the phosphorolytic pathway produces more ATP. However, an optimized engineered yeast having faster cellobiose transport and the phosphorolytic pathway consumes cellobiose as fast as the hydrolytic pathway through a thermodynamic "push" from the reactions to overcome the positive ΔG° of cellobiose phosphorolysis. Furthermore, energetic advantages are observed in stress conditions by the optimized phosphorolytic strain.

The disparity in free energy change may explain why additional genetic modifications were necessary to obtain high growth or fermentation rates in the phosphorolytic strains (Figs. 3 and 6). We propose that with a slightly positive ΔG°, the phosphorolytic cleavage of cellodextrins requires a thermodynamic "push" to drive the reaction forward (Fig. 7, Supplementary Fig. S14). Our data and known energetics of the reaction suggest that the rate of cellodextrin phosphorolysis is limited by low concentrations of substrate (cellobiose or longer cellodextrins) and unfavorable thermodynamics. From this perspective, the high-V_{max} transporter that emerged from our selection and other high-V_{max} transporters likely increase the cellodextrin consumption rate by raising intracellular cellodextrin concentrations. These higher intracellular concentrations would "push" the phosphorolytic reaction into conditions where ΔG is negative. Consistent with this hypothesis, the F213L high-V_{max} transporter improved the cellobiose utilization of yeast strains containing different CBPs (Fig. 3, Supplementary Fig. S1), and the cellodextrin utilization of a yeast strain containing CDP (Fig. 3), when compared to strains expressing the WT transporter (Figs. 2 and 3, Supplementary Fig. S1).

Neither of the two cellobiose fermentation pathways studied here are natural to *S. cerevisiae* and it is enlightening to consider their origins. The hydrolytic pathway consists of a high-affinity cellodextrin transporter and a β-glucosidase taken from the filamentous fungus, *N. crassa*. This pathway is relatively widespread and is found commonly in cellulolytic bacteria and eukaryotes (Galazka et al., 2010; Zhang and Lynd, 2004a). The phosphorolytic pathway shares cellodextrin transport but replaces the β-glucosidase with a cellobiose phosphorylase and/or cellodextrin phosphorylase (Parker and Hall, 1990). In contrast, this type of pathway seems to be limited almost entirely to bacteria, and all published examples of cellobiose and cellodextrin phosphorylases are bacterial (Alexander, 1968; Reichenbecher et al., 1997). To further investigate cellobiose utilization pathways, we reviewed the Carbohydrate-Active enZYmes Database (CAZy), which catalogues and classifies enzymes that act on sugars (Cantarel et al., 2009). Cellobiose phosphorylases are members of

Glycoside Hydrolase family 94 (GH94), along with cellodextrin phosphorylases, chitobiose phosphorylases and cyclic β-1,2-glucan synthases. At the present time, GH94 enzymes are heavily biased to being present in bacterial genomes in comparison to other GH families (Supplementary Fig. S13a). Whereas 97% of GH94s were bacterial while only 2% were eukaryotic, GH families 1 and 3, of which β-glucosidases are members, show a more even distribution. The CAZy database catalogs 83% of the GH1 and GH3 family enzymes as bacterial while 16% are eukaryotic. One way to assess the importance of these three families to the metabolism of bacteria and eukaryotes is to document the abundance of GH94 within each kingdom relative to the abundance of all three families (GH94/(GH1+GH3+GH94). This method has the advantage of correcting for the bias in available genomes. Using this approach shows that in bacteria, 7.4% of enzymes possibly active on cellobiose are members of GH94, while in eukaryotes only 0.5% are members of GH94 (Supplementary Fig. S13b). This 15-fold difference is strong evidence that phosphorolysis of cellodextrin is more widely used by bacteria than eukaryotes. Based on the data presented here, we propose that phosphorolysis of cellobiose is primarily advantageous under anaerobic conditions where there is strong selective pressure for pathways that maximize energy yield (Zhang and Lynd, 2005). In contrast, when oxygen is present the reversibility of the phosphorolytic reaction has too many disadvantages, and there is selection against it. Thus the possible absence of the phosphorolytic pathway in eukaryotes can be explained by the paucity of anaerobic eukaryotes that degrade cellulose. It will be interesting to determine whether anaerobic fungi found in ruminants (Hess et al., 2011) may also exploit the energetic benefits of phosphorolysis to consume the plant cell wall.

Author contributions

S.J.H. and J.M.G. contributed equally to this work. S.J.H., J.M.G., Y.S.J. and J.H.D.C. designed all experiments. E.J.O. and H.K. performed fermentation experiments and V.K. performed enzymatic assay experiments. S.J.H., J.M.G., Y.S.J. and J.H.D.C. analyzed data and wrote the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by funding from the Energy Biosciences Institute to Jamie Cate and Yong-Su Jin. The authors thank William Beeson, Soo Rin Kim, and Na Wei for helpful discussions, and Mara Bryan and Crystal Chan for technical support.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.11.005.

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