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

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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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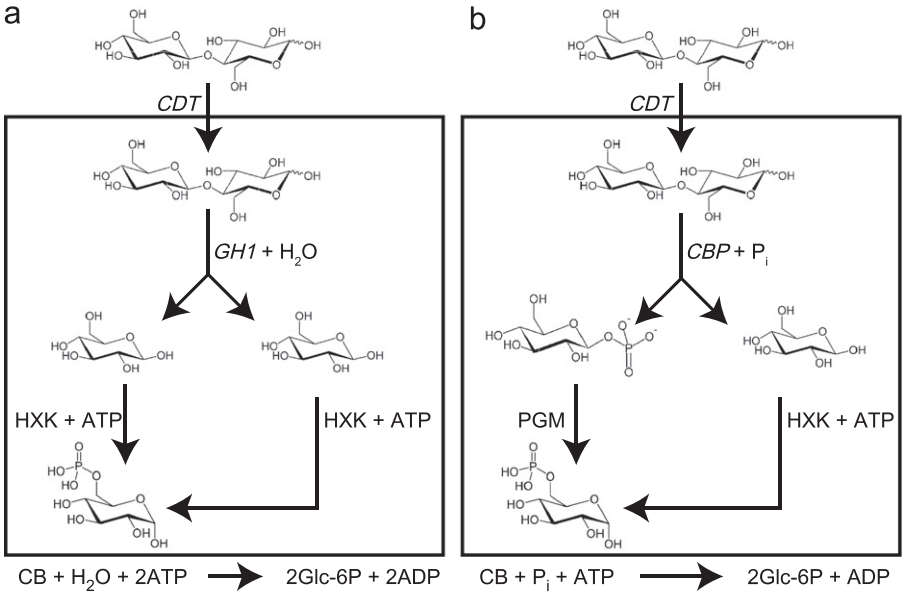

Fig. 1. Two possible cellobiose fermentation pathways. Following transport across the plasma membrane by a cellodextrin transporter (CDT), cellobiose is cleaved either (a) by hydrolysis *via* β-glucosidase (GH1 here, or GH3 (Galazka et al., 2010)) or (b) by phosphorolysis *via* cellobiose phosphorylase (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