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Author

Galazka, Jonathan Matthew

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Cellodextrin Transporters of *Neurospora crassa* and their Utility in *Saccharomyces cerevisiae* During a Biofuel Production Process.

By

Jonathan Matthew Galazka

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Committee in charge:

Professor Jamie H. D. Cate

Professor N. Louise Glass

Professor Randy Schekman

Professor Eva Nogales

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Abstract

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Professor Jamie H. D. Cate, Chair

The filamentous fungus, *Neurospora crassa*, is capable of depolymerizing and metabolizing plant cell walls. When grown on plant cell walls or pure cellulose, *N. crassa* upregulates an overlapping set of 114 genes. Amongst this set are 10 major facilitator superfamily transporters. I have shown that two of these, CDT-1 and CDT-2, transport cellodextrins, which are the major degradative product of fungal cellulases. Deletion of *cdt-2* affects the growth of *N. crassa* on crystalline cellulose. Furthermore, diverse fungi transcriptionally upregulate orthologs of *cdt-1* and *cdt-2* when in contact with plant cell walls, suggesting that cellodextrin transporters are important to fungal interactions with plants.

Engineering the cellodextrin transport pathway into *Saccharomyces cerevisiae* allows this yeast to ferment cellodextrins to ethanol with high yields, and facilitates the simultaneous saccharification and fermentation of cellulose to ethanol. Cellodextrin transport can be coupled to downstream hydrolysis or phosphorolysis of cellodextrins by a cellodextrin hydrolase or cellobiose phosphorylase, respectively. Cellodextrin transport circumvents a major limitation of yeast in fuel production: the inability to simultaneously transport and ferment pentose sugars and glucose to ethanol.

S. cerevisiae did not evolve to co-ferment cellobiose and xylose and unintended consequences are likely. For example, we speculate that *S. cerevisiae* may not sense cellobiose as a fermentable carbon source.

Dedication

I dedicate this dissertation to the friends, family members, teachers and colleagues that have made it possible. I thank my mother, Donna Marie Galazka, and my father, Sim Stevens Galazka, for their love, attention and confidence. I also thank my sister, Jessica Galazka Zarnegar, and my brother, Bryan Edmund Galazka for their companionship and solidarity. Finally, I thank my wife, Summer Kate Praetorius, who is my love, inspiration and unwavering partner.

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1. Introduction

1.1 Context and Motivation

Access to clean, reliable and affordable energy sources is intimately linked to human health and welfare. Increased energy supplies allow individuals to be more mobile and productive, and are strongly correlated to increased wealth and welfare in energy-poor nations (Martinez & Ebenhack, 2008). Therefore, expanding global energy production is critical to lifting people in poverty to acceptable living standards. In 2010, 33.6% of the energy consumed globally was derived from oil, 29.6% from coal, 23.8% from natural gas, 6.5% from hydroelectric, 5.2% from nuclear, and 1.3% from renewable sources (British Petroleum, 2011), with renewable sources defined as wind, geothermal, solar, biomass and municipal waste.

Consumption of all energy sources can be increased in the short term, but the expansion of renewables has become a priority because of environmental concerns. In 2007, the International Panel on Climate Change (IPCC) released its estimates for global temperature change assuming 6 separate greenhouse gas emission scenarios (IPCC, 2007). The increase in CO_2 emissions from the years 2000 (23.5 gigatons/year) to 2010 (30.6 gigatons/year) (IEA, 2011) is consistent with IPCC scenario A2, which is predicted to lead to an atmospheric CO_2 concentration of 850 parts per million by 2099, and which would raise the global average temperature by 2.0 – 5.4 °C. Furthermore, the IPCC predicts that a temperature increase of this magnitude would have numerous negative consequences on human society and natural ecosystems (IPCC, 2007).

Renewable sources of energy are therefore attractive because they can be produced with low net addition of CO_2 to the atmosphere. Energy produced from the combustion of biomass, either in its raw form or after it has been converted to a liquid fuel has net greenhouse gas emissions that are 60-90% lower than energy produced from the combustion of petroleum (Adler, Del Grosso, & Parton, 2007; Y. Bai, Luo, & van der Voet, 2010; Farrell et al., 2006; Wu, Wu, & Wang, 2006). Therefore, the development of an industry capable of producing significant quantities of liquid fuel from biomass has become a priority.

1.2 Lignocellulosic Biofuels

The term "lignocellulosic biofuels" describes any liquid fuel produced from plant cell walls (Carroll & Somerville, 2009). Fuels can be produced from plant cell walls using a number of methods. For example, it is possible to thermally convert plant cell walls to a gas stream composed of syn-gas (CO and H_2) as well as other gases such as CO_2, CH_4 and N_2. The technology for chemically converting syn-gas into a liquid fuel is well established (Huber, Iborra, & Corma, 2006). Another option is a bioconversion process relying first upon the enzymatic deconstruction of plant cell walls into soluble sugars. The free sugars are then converted to liquid fuels either chemically (Huber, Chheda, Barrett, & Dumesic,

2005), or *via* microbial fermentation by yeasts or bacteria (Stephanopoulos, 2007). Currently, none of these methods results in a market-competitive fuel (Aden & Foust, 2009; Sims, Mabee, Saddler, & Taylor, 2010), and technological barriers must be overcome (U. S. Dept. of Energy, 2006).

Plant cell walls

Lignocellulosic biofuels can be produced from agricultural, forestry or municipal waste, or from dedicated energy crops (U. S. Dept. of Energy, 2011). In all cases, fuels are produced from the energy-rich compounds that comprise the plant cell wall. Plant cell walls are a complex material consisting of cellulose, hemicellulose, pectin, lignin and highly glycosylated proteins (Carpita & Gibeaut, 1993; Fry, 2004; Somerville et al., 2004). Cellulose is a β-1,4-linked glucose polymer commonly hundreds to thousands of glucans long. Within plant cell walls, cellulose is found in crystalline microfibrils consisting of 30 – 36 cellulose polymers associated by intermolecular hydrogen bonds. Hemicellulose describes a heterogeneous set of branched polymers consisting of pentoses, hexoses, and sugar acids that form hydrogen bonds to cellulose and other hemicellulose polymers (Scheller & Ulvskov, 2010). Pectins are a complex family of polysaccharides that contain 1,4-linked galactouronate (Ridley, O'Neill, & Mohnen, 2001). Lignin is a heterogeneous phenolic polymer distributed throughout the plant cell wall that imparts considerable rigidity to plant cell walls. The monomeric constituents of lignin are coumaryl, coniferyl and sinapyl alcohols (Achyuthan et al., 2010). Heavily glycosylated proteins reinforce the polysaccharide network, but are present in plant cell walls in small amounts compared to polysaccharides and lignin (Albenne et al., 2009).

The exact composition of plant cell walls varies between plant species, and in different tissues and developmental stages within a plant species (Pauly & Keegstra, 2010). Corn stover, the material that remains after the cereal grains of *Zea mays* are harvested, is being considered as a source of waste biomass for lignocellulosic biofuel production. It consists of 37.7% cellulose, 28.3% hemicellulose (21.6% xylan, 2.4% arabinan, 0.9% galactan, 0.4% mannan), and 18.6% lignin (Carroll & Somerville, 2009). Switchgrass (alamo strain) is being considered as a dedicated energy crop. In comparison, it consists of 31% cellulose, 24.4% hemicellulose (20.4% xylan, 2.8% arabinan, 0.9% galactan, 0.3% mannan), and 17.6% lignin (Carroll & Somerville, 2009).

Industrial plant cell wall deconstruction

To release the constituent sugars from plant cell walls, industrial processes commonly rely on mechanical, thermal, chemical and enzymatic processes. The National Renewable Energy Laboratory (NREL) has described a "dilute acid pretreatment and enzymatic hydrolysis of corn stover" process that consists of the following steps (Humbird, 2011). Dry biomass is mechanically reduced in size and then treated with dilute sulfuric acid at high temperatures for a short period of time to release hemicellulosic sugars and other compounds. Cellulose is refractory to this treatment, and is subsequently

depolymerized by cellulases and other enzymes. Cellulases catalyze the hydrolysis of cellulose by acting either on the ends of a cellulose chain (exoglucanases) or within a cellulose chain (endoglucanases), and work in concert with a number of accessory enzymes. These accessory enzymes include the oxidative enzymes, cellobiose dehydrogenase and polysaccharide monooxygenase (Phillips, Beeson, Cate, & Marletta, 2011). Cellulolytic fungi (Dashtban, Schraft, & Qin, 2009), bacteria (Maki, Leung, & Qin, 2009) and a limited number of crustaceans (King et al., 2010), produce cellulases and accessory enzymes, but the NREL process relies upon those produced by the fungus, *Hypocrea jecorina* (*Trichoderma reesei*) (Seidl et al., 2008). Treatment of cellulose by cellulases produces short oligosaccharides of β-1,4-linked glucose called cellodextrins, which are further hydrolyzed to glucose by β-glucosidases (Xin, Yinbo, & Peiji, 1993).

Plant cell wall conversion

Once sugars are liberated from plant cell walls, the soluble hexoses and pentoses can be converted to liquid fuels either chemically, or *via* microbial fermentation by yeasts or bacteria (Weber et al., 2010). The chemical processes include zeolite upgrading to hydrocarbons and aqueous phase processing to alkanes (Huber, 2006). Microbial fermentation can be performed with a number of organisms including the bacteria, *Zymomonas mobilis* and *Clostridia acetobutylicum,* and the yeasts, *Saccharomyces cerevisiae* and *Pichia stipitis (Scheffersomyces stipitis)*. Fermentation byproducts suitable as fuels include ethanol and butanol. The most common processes currently utilize *S. cerevisiae* or *Z. mobilis* to ferment hexoses to ethanol (Weber, et al., 2010).

1.3 Fungi

The Kingdom Fungi contains approximately 1.5 million species of yeasts, molds, mushrooms, lichens, rusts and smuts that are important to natural ecosystems and human societies (Stajich et al., 2009). Fungi play a key role in nutrient cycling by degrading organic matter, and by aiding nutrient uptake by plants. Fungi are alternately beneficial and harmful to humans, having been used as a source of nutrition, pharmaceuticals and chemicals, but also causing disease, crop destruction and property damage. Fungi are morphologically defined by a cell wall made of chitin (β-1,4 linked *N*-acetylglucosamine), by their filamentous growth pattern, and by their multinucleate cells, although exceptions exist (Alexopoulos, 2007). The kingdom is traditionally divided into 4 phyla: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota. Only the latter two are considered monophyletic. The Kingdom Fungi is overwhelmingly dominated by two phyla; approximately 65% and 35% of described fungal species fall within Ascomycota and Basidiomycota, respectively (Ainsworth, Bisby, Kirk, & Bioscience, 2008).

Lignocellulolytic fungi

In natural ecosystems, fungi have an important role in the recycling of organic material bound within plants, both living and dead. Those that degrade non-living plant matter are classified as saprophytes, while those that consume living material are endophytes (no negative consequences to the host plant) or parasites (negative consequences to the host plant) (Alexopoulos, 2007). Fungi degrade plant material by secreting variety of degradative enzymes. These include lignin-active enzymes (e.g. lignin peroxidases and manganese peroxidases), hemicellulose-active enzymes (e.g. endoxylanases, glucuronidases and arabinofuranosidases) and cellulose active enzymes (e.g. cellobiohydrolases and endocellulases). The concerted action of these enzymes reduces plant material to its constituent monomers or short polymers, which can be imported by specific nutrient transporters for intracellular metabolism (Dashtban, et al., 2009).

Neurospora crassa

Neurospora crassa is a member of the Ascomycota (R. H. Davis & Perkins, 2002). It is commonly found in tropical, sub-tropical and temperate regions (Perkins & Turner, 1988) where it is one of the first organisms to colonize dead and damaged plants in fire-affected areas (Pandit & Maheshwari, 1996). The lifecycle of *N. crassa* is typical of the Ascomycota. It spends most of it life in a haploid state, with vegetative growth forming a dense, interconnected network of hyphae. In response to specific environmental cues, multinucleate macroconidia and mononucleate microconidia will form, be released into the environment and germinate to form a new haploid colony. The sexual cycle of *N. crassa* begins when reproductive structures of the opposite mating type (*a* and *A*) come into contact. Then their cell walls breakdown, and plasma membrane and nuclei fusion occurs. These diploid nuclei undergo a round of meiosis followed by a round if mitosis to form eight haploid ascospores, all contained within one ascus. After being ejected from the ascus, each ascospore will develop into a haploid hyphal colony, completing the sexual cycle (Rowland H. Davis, 2000).

The cellulolytic capacity of *N. crassa* was first described in 1949 by Marsh *et al.* (Marsh, Bollenbacher, Butler, & Raper, 1949), and a specific induction system for the *N. crassa* cellulase system was developed in 1977 by Eberhart *et al*. (Eberhart, Beck, & Goolsby, 1977). In 1990, three exoglucanases, four endoglucanases, and one β-glucosidase were purified from the *N. crassa* secretome (Yazdi, Radford, Keen, & Woodward, 1990), and in 1995 the gene encoding cellobiohydrolase I (*cbh-1*) was cloned (Taleb & Radford, 1995). Study of the *N. crassa* cellulases system stopped until 2010. At this point, a systems-level analysis of plant cell wall metabolism by *N. crassa* was performed, establishing that an overlapping set of 114 genes were induced in response to plant cell walls or pure cellulose (Tian et al., 2009). Within this set were cellulases and other plant cell wall-active enzymes, but also a variety of genes involved in diverse cellular processes including nutrient transport, metabolism, and protein secretion. Quantitative proteomics established that 63 - 65% of the total secretome of *N. crassa* grown on pure cellulose consists of two cellobiohydrolases (CBH-I, GH6-2), an endoglucanase (GH5-1) and a β-glucosidase(GH3-4). However, when used at their natural abundance, these enzymes only accounted for 43% of

the total degradative capacity of the complete *N. crassa* secretome (Phillips, Iavarone, & Marletta, 2011). This suggests that efficient plant cell wall degradation by *N. crassa* requires a complex suite of enzymes. The partial roles for three enzymes: a lactonase (Beeson, Iavarone, Hausmann, Cate, & Marletta, 2011), cellobiose dehydrogenase and a polysaccharide monooxygenase (Phillips, Beeson, et al., 2011) have been proposed.

Ethanologenic yeast

The term "yeast" describes any unicellular fungus and does not describe a monophyletic group. Budding yeasts within the phylum Ascomycota form a sister group (subphylum Saccharomycotina) with filamentous ascomycetes (subphylum Pezizomycotina), while fission yeasts within Ascomycota form a clade basal to both groups (class Schizosaccharomycetes within subphylum Taphrinomycotina). Yeasts are found within all three major lineages of Basidiomycota (Agaricomycotina, Pucciniomycotina, Ustilaginomycotina) (Kurtzman & Piskur, 2006). Yeasts have been used by human societies for thousands of years because of their capacity to ferment sugars to ethanol and CO_2 (Pretorius, 2000). Reports of wild tree shrews habitually consuming fermented nectar suggest that this relationship between yeasts and primates may not be exclusive to humans (Wiens et al., 2008).

Saccharomyces cerevisiae

S. cerevisiae is the prototypical yeast. It is naturally associated with rotting fruit and other sources of soluble sugars, and is used by human societies for its ability to produce ethanol and CO_2 from soluble sugars (Liti et al., 2009). *S. cerevisiae* has a number of traits that make it particularly useful to human societies, including the capacity to produce ethanol in the presence of oxygen and the ability to grow anaerobically. These traits are thought to have arose following a whole-genome duplication event (Wolfe & Shields, 1997), and a series of horizontal gene-transfer events (Butler et al., 2004; Gojkovic et al., 2004; Keeling & Roger, 1995).

Natural physiology and metabolism of *S. cerevisiae*

S. cerevisiae has minimal nutritional requirements, and is commonly grown in a media consisting of salts plus biotin, pantothenate, folic acid, inositol, niacin, p-aminobenzoic acid, pyridoxine, riboflavin, and thiamine, plus a source of reduced carbon (Sherman, 2002). *S. cerevisiae* is capable of utilizing glucose, fructose, sucrose, galactose, melbiose, maltose, ethanol, lactate, and glycerol as carbon sources. Growth on non-fermentable carbon sources requires oxygen. Despite this flexibility, *S. cerevisiae* is best known for its capacity to ferment glucose to ethanol at very high rates (Lau, Gunawan, Balan, & Dale, 2010). The metabolic pathway responsible for this begins with the transport of glucose into the cell by one of sixteen hexose transporters (Lagunas, 1993). Glucose is then converted to two molecules of pyruvate by the concerted action of the enzymes in the

Embden-Meyerhoff glycolytic pathway. Pyruvate is converted to ethanol and CO_2 by the action of pyruvate decarboxylase and alcohol dehydrogenase (Rodrigues, Ludovico, & Leao, 2006). Alternatively pyruvate can be converted to acetyl-cofactor A and CO_2 by mitochondrial pyruvate dehydrogenase, or cytosolic pyruvate decarboxylase and acetaldehyde dehydrogenase. Mitochondrial acetyl-cofactor A is converted to CO_2 and H_2O by the tricarboxylic acid and oxidative phosphorylation pathways (Rodrigues, et al., 2006).

S. cerevisiae engineered to ferment xylose

S. cerevisiae does not naturally consume or ferment xylose, a major component of plant cell walls (Kim, Block, & Mills, 2010a). This limits its usefulness as an ethanologen in a lignocellulosic biofuels process. To overcome this limitation, *S. cerevisiae* has been engineered to express xylose reductase and xylitol dehydrogenase, usually from *Scheffersomyces stipitis* (Karhumaa, Garcia Sanchez, Hahn-Hagerdal, & Gorwa-Grauslund, 2007; Kotter, Amore, Hollenberg, & Ciriacy, 1990). The concerted action of these enzymes converts xylose to xylulose. Xylulose is a substrate of the *S. cerevisiae* pentose phosphate pathway and can be fermented to ethanol. Additional modifications are necessary for rapid fermentation of xylose, including the overexpression of enzymes within the non-oxidative branch of the pentose phosphate pathway (xylulose kinase, transaldolase, transketolase) (Karhumaa, Hahn-Hagerdal, & Gorwa-Grauslund, 2005) and deletion of the aldose reductase, *GRE3* (Traff, Otero Cordero, van Zyl, & Hahn-Hagerdal, 2001). An alternate strategy of adding a bacterial xylose isomerase to *S. cerevisiae* has been successful as well, but the xylose fermentation rates of these strains is low (Kuyper et al., 2003).

Xylose enters these engineered *S. cerevisiae* strains through the native sugar transport system (Hamacher, Becker, Gardonyi, Hahn-Hagerdal, & Boles, 2002). While a single step in a metabolic pathway is never rate limiting (Kacser & Burns, 1973), the rate of xylose transport has an important affect on the overall rate of xylose fermentation (Gardonyi, Jeppsson, Liden, Gorwa-Grausland, & Hahn-Hagerdal, 2003; Kuyper et al., 2005; A. Saloheimo et al., 2007). To improve xylose transport rates, *S. cerevisiae* has been engineered to express various sugar transporters with some success (Leandro, Goncalves, & Spencer-Martins, 2006; Runquist, Fonseca, Radstrom, Spencer-Martins, & Hahn-Hagerdal, 2009; Young, Lee, & Alper, 2010). A remaining problem is that both the native sugar transporters of *S. cerevisiae*, and the recombinant transporters that have been tested, have a higher affinity for glucose than xylose. For example, there are two native transport systems in *S. cerevisiae*, a high-affinity system and a low-affinity system. The high-affinity system has a glucose K_m of $\sim$1.5 mM but a xylose K_m of 190 mM. The low-affinity system has a glucose K_m of $\sim$20 mM but a xylose K_m of 1.5 M (Kotter & Ciriacy, 1993). This is problematic during fuel and chemical production from lignocellulose, as plant cell walls contain both sugars. Systematic searches for transporters that can be expressed in yeast, and that strongly prefer xylose to glucose, have not been successful (Young, Poucher, Comer, Bailey, & Alper, 2011).

Fermentation of cellodextrins by *S. cerevisiae*

While *S. cerevisiae* rapidly ferments glucose to ethanol, it is naturally incapable of fermenting cellodextrins such as cellobiose. Cellodextrins are the short β-1,4-linked oligosaccharides of glucose that make up cellulose, and are the primary product of cellulases (Lynd, Weimer, van Zyl, & Pretorius, 2002b). To overcome this limitation, *S. cerevisiae* has been engineered to produce secreted β-glucosidases. This was first accomplished in 1986 by Kohchi and Toh-e (Kohchi & Toh-e, 1986) and has been repeated numerous times since (Benoliel, Pocas-Fonseca, Torres, & de Moraes, 2010; Kotaka et al., 2008b; Murai, Ueda, Kawaguchi, Arai, & Tanaka, 1998; Rajoka et al., 1998; Saitoh, Tanaka, & Kondo, 2008; Shen et al., 2008; Tokuhiro, Ishida, Kondo, & Takahashi, 2008; Uryu et al., 2006; Van Rensburg, Van Zyl, & Pretorius, 1998; van Rooyen, Hahn-Hagerdal, La Grange, & van Zyl, 2005).

2. Cellodextrin transport systems of *Neurospora crassa* and their recapitulation in *S. cerevisiae* for improved biofuels

Partially taken from: Galazka, J. M., Tian, C., Beeson, W. T., Martinez, B., Glass, N. L., & Cate, J. H. (2010). Cellodextrin transport in yeast for improved biofuel production. *Science*, *330*(6000), 84-86.

2.1 Abstract

Fungal degradation of plant biomass may provide insights for improving cellulosic biofuel production. We show that the model cellulolytic fungus *Neurospora crassa* relies on a high-affinity cellodextrin transport system for rapid growth on cellulose. Reconstitution of the *N. crassa* cellodextrin transport system in *Saccharomyces cerevisiae* promotes efficient growth of this yeast on cellodextrins. In simultaneous saccharification and fermentation experiments, the engineered yeast strains more rapidly convert cellulose to ethanol when compared with yeast lacking this system.

2.2 Results and Discussion

In 2009, a systematic analysis of plant cell wall degradation by the model cellulolytic fungus, *Neurospora crassa*, revealed that it increases transcription of 10 major facilitator superfamily (MFS) transporters when grown on pure cellulose (Tian, et al., 2009). A phylogenetic analysis of these transporters demonstrates that seven of the transporters cluster into two distinct clades of transporters. The first clade contains NCU00801, NCU08114 and NCU05853 (**Figure 2-1, Red**). All of these are annotated as sugar transporters. The second clade contains NCU04963 and NCU10021 (annotated as sugar transporters), and NCU06138 and NCU0988 (annotated as quinate transporters) (**Figure 2-1, Blue**). NCU05897, NCU01231 and NCU05519 are more distantly related, and are annotated as an l-fucose transporter, a carboxylic acid transporter and transporter of undefined function, respectively. A phylogenetic tree constructed with these transporters plus related *N. crassa* genes (NCU07199, NCU11342, NCU00809, NCU10021), the closest *Chaetomium globosum* homolog of each *N. crassa* gene, the 16 hexose transporters of *S. cerevisiae* (Hxt1 – Hxt17), and the four hexose transporters of *P. stiptis* (Hxt2.1 – Hxt2.4) is shown in **Figure 2-2**. The clustering of NCU04963, NCU10021, NCU00988, and NCU06138 with the *S. cerevisiae* hexose transporters may suggest that these *N. crassa* genes share a similar function: to import hexose sugars (Lagunas, 1993). The function of the *P. stiptis* hexose transporters is not fully understood, but a recent patent filing supports the idea that some of them may act as cellobiose transporters (T. Jeffries, Nelson, & Mahan, 2011).

In addition to the 10 MFS sugar transporters, *N. crassa* also induces a β-glucosidase (NCU00130) that is predicted to be intracellular (Galagan et al., 2003; Tian, et al., 2009). NCU00130 is a family 1 glycoside hydrolase, and is predicted to catalyze the hydrolysis of short cellodextrins into glucose (Cantarel et al., 2009). A homologous enzyme has been identified and cloned from the related fungus, *Trichoderma reesei* (*Hypocrea jecorina*) (M.

Saloheimo, Kuja-Panula, Ylosmaki, Ward, & Penttila, 2002). It was shown to be intracellular, and to be active on cellobiose, cellotriose and cellotetraose. A mass spectrometry-based analysis of the secretome of *N. crassa* growing of pure cellulose did not detect any peptides from NCU00130, supporting its intracellular location (Tian, et al., 2009).

The induction of an intracellular β-glucosidase (NCU00130) in concert with predicted sugar transporter in response to cellulose but not sucrose (Tian, et al., 2009), suggested that metabolism of cellulose by *N. crassa* involves the intracellular hydrolysis of cellodextrins. It also suggested that the cellodextrins were entering the cytosol of *N. crassa* through one of the 10 induced transporters.

To test these hypotheses we assayed the growth, and sugar-consumption of *N. crassa* single gene deletion strains. A strain carrying a deletion for NCU08114, grew slowly on cellulose (**Figure 2-3**), and strains lacking either NCU08114 or NCU00801 consumed cellobiose, cellotriose, and cellotetraose poorly (**Figure 2-4**). Orthologs of NCU08114 and NCU00801 are widely distributed in the fungal kingdom (**Figure 2-5**), and recent whole-genome transcriptional profiling studies show their importance to interactions between fungi and plants. For example, some cellulolytic fungi increase expression levels of NCU08114 orthologs while degrading plant wall material (Noguchi et al., 2009), and the Périgord black truffle increases the expression of a NCU00801 ortholog during symbiotic interactions with plant roots (Martin et al., 2010). In addition, certain yeasts that grow on cellobiose contain orthologs of NCU08114 and NCU00801 (S. N. Freer, 1991). All of these organisms also contain genes for intracellular β-glucosidases (**Table 2-6**), suggesting that cellodextrin transport systems are widespread in nature and are essential for optimal growth of fungi on cellulose-derived sugars.

Since cellobiose is not catabolized by *S. cerevisiae* (Lynd, Weimer, van Zyl, & Pretorius, 2002a; Sun & Cheng, 2002), and is not accumulated in the cytoplasm (**Figure 2-7**), we reasoned that expression of a functional cellodextrin transport system from *N. crassa* might allow *S. cerevisiae* to grow with cellobiose as the sole carbon source. To test this idea, *S. cerevisiae* was transformed with a plasmid containing the NCU00130 open reading frame. This strain was then transformed individually with 7 out of the 10 cellulose-induced MFS transporters, also carried on a plasmid (NCU10021, NCU05519 and NCU5897 were not tested because of cloning difficulties). When assayed for growth with cellobiose as a sole carbon source, strains bearing NCU00801 and NCU08114 grew rapidly, with doubling times of ~30% and ~12% of the doubling time with glucose as a carbon source, respectively (**Figure 2-8, Figure 2-9**).

Notably, cellodextrins longer than cellobiose also support the growth of yeast expressing these transporters (**Figure 2-9**), indicating that cellodextrins longer than cellobiose are transported by NCU00801 and NCU08114, hereafter called CDT-1 and CDT-2. Growth cannot be explained by the extracellular hydrolysis of cellodextrins to glucose followed by transport, as a strain expressing only the putative intracellular β-glucosidase, GH1-1, grew slowly on cellodextrins (**Figure 2-8, Figure 2-9**). Furthermore, the activity of GH1-1, which is able to hydrolyze cellobiose, cellotriose and cellotetraose (**Figure 2-10**), is

negligible in culture supernatants, and cannot explain the growth of yeast expressing the transporters (**Figure 2-11**).

To directly assay transporter function, the uptake of [^{3}H]-cellobiose into yeast cells was measured. Both CDT-1 and CDT-2 are high-affinity cellobiose transporters, with K_m values of 4.0 ± 0.3 μM and 3.2 ± 0.2 μM (mean ± SD), respectively (**Figure 2-12**). The expression-normalized V_{max} of CDT-1 is over twice that of CDT-2, which might explain differences in the observed growth rates of the yeast strains (**Figure 2-8, Figure 2-9**). Notably, cellobiose transport by CDT-1 and CDT-2 is inhibited by excess cellotriose, and CDT-1 activity is also inhibited by cellotetraose (**Figure 2-13**). These data further support that CDT-1 and CDT-2 directly transport the longer cellodextrins in growth assays (**Figure 2-9**). Thus, the combinations of CDT-1 or CDT-2 with GH1-1 constitute fully functional cellodextrin transport systems.

MFS sugar transporter can either act as uniporters, symporter or antiporters. Uniporters equilibrate the concentration of one substrate across the plasma membrane. Symporters and antiporters equilibrate the concentration of two substrates across the plasma membrane. A complete transport cycle of a symporter moves both substrates to one side of the membrane, while that of an antiporter moves the two substrates to opposite sides (Law, Maloney, & Wang, 2008). In fungi, a common form of symport takes advantage of the strong proton gradient that is maintained across the plasma membrane. In *S. cerevisiae*, this gradient is maintained by Pma1p, an ATPase that moves ~1 proton out of the cell at the expense of ~1 intracellular ATP (Miranda, Pardo, & Petrov, 2011). The *N. crassa* ortholog is PMA-1, and has similar properties (Kuhlbrandt, Zeelen, & Dietrich, 2002). To test if CDT-1 or CDT-2 utilize the plasma membrane proton gradient during their transport cycle, we measured the sensitivity of [^{3}H]-cellobiose transport to carbonyl cyanide-m-chlorophenylhydrazone (CCCP). CCCP intercalates into the plasma membrane and makes it porous to protons (Stevens & Nichols, 2007). The CDT-1-mediated transport rate of cellobiose is reduced in response to increasing concentrations of CCCP, while the CDT-2-mediated rate is not (**Figure 2-14**).

We next tested whether, in addition to growth, a complete cellodextrin catabolism pathway would be useful in *S. cerevisiae* for lignocellulosic biofuel production. With little optimization, yeast expressing *cdt-1* and *gh1-1* fermented cellobiose with an ethanol yield of 0.44 (g ethanol/g glucose), 86.3% of the theoretical value (**Figure 2-15 A**) (F. W. Bai, Anderson, & Moo-Young, 2008). This yield is close to present industrial yields of ethanol from glucose of 90 – 93% (Basso, de Amorim, de Oliveira, & Lopes, 2008).

Fungal cellulases evolved to function in conjunction with the consumption of sugars released from plant cell walls, and work most effectively in this context (Bhat & Maheshwari, 1987). Simultaneous saccharification and fermentation (SSF) mimics this natural synergy by the addition of fermenting microbes to the plant biomass depolymerization reaction, resulting in a more efficient process (Doran-Peterson et al., 2009; Drissen, Maas, Tramper, & Beeftink, 2009). Current SSF schemes are limited by the need for quantitative conversion of cellulose to glucose outside yeast cells (Xin, et al., 1993). A cellodextrin transport system could be particularly useful during SSF, as it would

remove the requirement for full hydrolysis of cellulose to glucose by extracellular β-glucosidases (Chauve et al., 2010), and would reduce the risk of contamination by glucose-dependent organisms (Skinner & Leathers, 2004). Furthermore, both CDT-1 and CDT-2 have a higher apparent affinity for cellobiose ($K_m \approx 3$ - 4 μM, **Figure 2-12**) when compared to secreted fungal β-glucosidases ($K_m \approx 100 - 1000$ μM (Chauve, et al., 2010)) and to *S. cerevisiae* hexose transporters' apparent affinity for glucose ($K_m \approx 1000 - 10{,}000$ μM (Reifenberger, Boles, & Ciriacy, 1997)). Therefore, the cellodextrin transport systems should more effectively maintain soluble sugar levels below the concentration at which they inhibit fungal cellulases (K_I of cellobiose ≈ 19 – 410 μM (Voutilainen et al., 2008)). Yeasts expressing a cellodextrin transport system markedly improve the efficiency of SSF reactions by reducing the steady state concentration of both cellobiose and glucose, and by increasing the ethanol production rate (**Figure 2-15 B & C**).

The addition of a cellodextrin transport system to biofuel-producing strains of yeast (**Figure 2-16**) overcomes a major bottleneck to fermentation of lignocellulosic feedstocks and likely will help to make cellulosic biofuels economically viable.

2.3 Materials and Methods

Strains

The *S. cerevisiae* strain used in this study was YPH499 (Sikorski & Hieter, 1989), which has the genotype: *MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δhis3-Δ200 leu2-Δ1*. It was grown in YPD media supplemented to 100 mg/L adenine hemisulfate. Transformed strains (Becker & Lundblad, 2001) were grown in the appropriate complete minimal dropout media, supplemented to 100 mg/L adenine hemisulfate. *Neurospora crassa* stains used in this study were obtained from the Fungal Genetics Stock Center (FGSC) (McCluskey, 2003) and include WT (FGSC 2489) and two cellobiose transporter deletion strains (FGSC 16575, ΔNCU00801.2 and FGSC 17868, ΔNCU08114.2 (Colot et al., 2006)).

Plasmids and Cloning

Transporters were cloned into the 2μ plasmid, pRS426, which was modified to include the *S. cerevisiae* PGK1 promoter inserted between SacI and SpeI using the primers, ATATATGAGCTCGTGAGTAAGGAAAGAGTGAGGAACTATC and ATATATACTAGTTGTTTTATATTTGTTGTAAAAAGTAGATAATTACTTCC (In all primers above and below, restriction sites are underlined). NCU00801 with a C-terminal Myc-tag and optimized Kozak sequence (Miyasaka, 1999) was then inserted between BamHI and EcoRI using the primers, ATGGATCCAAAAATGTCGTCTCACGGCTCC and ATGAATTCCTACAAATCTTCTTCAGAAATCAATTTTTGTTCAGCAACGATAGCTTCGGAC, and NCU08114 with a C-terminal Myc-tag and optimized Kozak sequence was inserted between SpeI and ClaI using the primers, ATACTAGTAAAAATGGGCATCTTCAACAAGAAGC and GCATATCGATCTACAAATCTTCTTCAGAAATCAATTTTTGTTCAGCAACAGACTTGCCCTCATG. To make GFP fusions, superfolder GFP (Pedelacq, Cabantous, Tran, Terwilliger, & Waldo, 2006) with an N-terminal linker of Gly-Ser-Gly-Ser was first inserted between the ClaI and SalI sited of the PGK1 promoter-containing pRS426 plasmid with the primers,

TATTAAATCGATGGTAGTGGTAGTGTGAGCAAGGGCGAGGAG and TATTAAGTCGACCTACTTGTACAGCTCGTCCATGCC. Transporters were then fused to GFP as follows: NCU00801 was inserted between BamHI and EcoRI using the primers, GCATGGATCCATGTCGTCTCACGGCTCC and TATAATGAATTCAGCAACGATAGCTTCGGAC, and NCU08114 was inserted between SpeI and EcoRI using the primers, TATTAAACTAGTATGGGCATCTTCAACAAGAAGC and TTATAAGAATTCAGCAACAGACTTGCCCTCATG.
The β-glucosidase, NCU00130, was cloned into the 2μ plasmid, pRS425, modified to include the PGK1 promoter described above. NCU00130 with an optimized Kozak sequence and a C-terminal 6xHis tag was inserted between SpeI and PstI using the primers, GCATACTAGTAAAAATGTCTCTTCCTAAGGATTTCCTCT and ATACTGCAGTTAATGATGATGATGATGATGGTCCTTCTTGATCAAAGAGTCAAAG. All constructs included the Cyc transcriptional terminator between XhoI and KpnI. All *N. crassa* genes were amplified by PCR from cDNA that was synthesized from mRNA isolated from *N. crassa* (FGSC 2489) cultured on minimal media with pure cellulose (Avicel) as the sole carbon source.

Yeast Growth Assays

To monitor growth on cellodextrins, engineered strains were grown in 5 mL of complete minimal media with appropriate dropouts, overnight. These starter cultures were washed 3x with 25 mL of ddH2O, and resuspended to an OD (600 nm) of 0.1 in Yeast Nitrogen Base (YNB) plus the appropriate Complete Supplemental Media (CSM) and 1% (w/v) cellobiose, or 0.5% (w/v) of either cellotriose, or cellotetraose. Assays were performed in a Bioscreen C™ with constant shaking at maximum amplitude at 30 °C, and a final assay volume of 0.4 mL. The change in OD was measured either at 600 nm or using a wideband filter from 450 – 580 nm. Growth rates were taken from the linear portion of each growth curve, and are reported as the mean of three independent experiments ± the standard deviation between these experiments. Cellotriose and cellotetraose was obtained from Seikagaku Biobusiness Corporation.

Purification of NCU00130 (named GH1-1) and assay of its activity

A 1 L culture of *S. cerevisiae* expressing *cdt-1* and *gh1-1* was grown to an OD (600 nm) of 2.0 in complete minimal media. Cells were harvested by centrifugation, and resuspended in 30 mL of lysis buffer (50 mM NaH2PO4 [pH 8.0], 300 mM NaCl, 10 mM imidazole, 2 mM β-ME, Complete™ Mini, EDTA free protease inhibitor cocktail). Cells were lysed by sonication, and the lysate cleared by centrifugation at 15,000 g for 30 minutes. The lysate was bound to 1 mL of Ni-NTA resin by gravity flow, and washed 3x with 25 mL wash buffer (Identical to lysis buffer but with 20 mM imidazole). GH1-1 was eluted with 5 mL of elution buffer (Identical to lysis buffer but with 250 mM imidazole), and the appropriate fractions were pooled, exchanged into storage buffer (Phosphate Buffered Saline, 2 mM DTT, 10% glycerol), aliquoted, frozen in liquid nitrogen, and stored at -80 °C. Purity was determined by SDS-PAGE, and protein concentration determined from the absorbance at 280 nm, using an extinction coefficient of 108750 M^{-1} cm^{-1}. Purified GH1-1 was assayed for hydrolysis activity with different cellodextrin substrates. Activity was measured by incubating 5 pmol of enzyme with 500 μM of each sugar in 150 μL Phosphate Buffered Saline (PBS) plus 3 mM DTT. Reactions proceeded for 40 minutes at 30 °C, before 100 μL

was removed and quenched in 400 µL of 0.1 M NaOH. The results were analyzed by ion chromatography with a Dionex ICS-3000, using a CarboPac PA200 column. Peaks were detected with an electrochemical detector.

Phylogenetic Analysis of Transporter Orthologs

Amino acid sequences of orthologs of *cdt-1* and *cdt-2* were obtained from online databases. Multiple sequence alignments were performed using T-Coffee (Notredame, Higgins, & Heringa, 2000). A maximum likelihood phylogeny was determined using PhyML version 3.0 (Guindon & Gascuel, 2003) with 100 bootstraps. Both programs were accessed through Phylogeny.fr (http://www.phylogeny.fr/). The resulting tree was visualized with FigTree v.1.2.1 (http://tree.bio.ed.ac.uk/).

Fermentation and Simultaneous Saccharification and Fermentation (SSF)

In fermentation and SSF experiments, comparisons were made between yeast expressing *gh1-1* and either Myc-tagged *cdt-1*, or no transporter. These strains were grown aerobically overnight in complete minimal media, washed 3x with 25 mL water, and resuspended to a final OD (600 nm) of 2.0 in 50 mL YNB plus the appropriate CSM, and either 2% (w/v) cellobiose or 3% (w/v) pure cellulose (Avicel), in sealed serum flasks. The SSF reactions also included 50 Filter Paper Units/g cellulose of filter-sterilized Celluclast (Sigma C2730), without β-glucosidase supplementation. Reactions were carried out anaerobically at 30 °C with shaking. At indicated time points, 1 mL samples were removed and filtered through a 0.2 µm syringe filter. The ethanol, glucose, and cellobiose concentrations in the filtrate were determined by HPLC with an Aminex 87H column and refractive index detection.

N. crassa Growth and Alamar Blue® Assays

WT *N. crassa* (FGSC 2489), and the homokaryotic NCU08114 (FGSC 17868) deletion strain (Colot, et al., 2006) were acquired from the FGSC (McCluskey, 2003), and grown at 25 °C in 50 mL of Vogel's salts plus 2% (w/v) of either sucrose or pure cellulose (Avicel) in a 250 mL unbaffled flask using an inoculum of 10^6 conidia/mL. After 16 or 28 hours, respectively, 100 µL of Alamar Blue® was added, and cultures incubated at room temperature for 20 minutes. At this time, 1 mL samples were removed, debris pelleted, and the fluorescence of 100 µL of the supernatant determined with excitation/emission wavelengths of 535/595 nm in a Beckman Coulter Paradigm plate reader.

N. crassa Cellobiose Transport Assays

WT *N. crassa* (FGSC 2489), and homokaryotic deletion lines (Colot, et al., 2006) of NCU00801 (FGSC 16575) and NCU08114 (FGSC 17868) were acquired from the FGSC (McCluskey, 2003), and grown for 16 hours in 50 mL Vogel's salts plus 2% (w/v) sucrose at 25 °C, starting with an inoculum of 10^6 conidia/mL. Mycelia were harvested by centrifugation, washed 3x with Vogel's salts, and transferred to Vogel's salts plus 0.5% (w/v) pure cellulose (Avicel) for 4 hours to induce the transporter expression. 10 mL of the culture was harvested by centrifugation, washed 3x with Vogel's salts, and resuspended in 1 mL ddH_2O plus cycloheximide (100 µg/mL) and 90 µM of the respective cellodextrin. To measure cellodextrin consumption, 100 µL was removed after 15 minutes, clarified by

centrifugation, and transferred into 900 µL of 0.1 M NaOH. The amount of sugar remaining in the supernatant was determined by HPLC with a Dionex ICS-3000, using a CarboPac PA200 column. Peaks were detected with an electrochemical detector.

GFP Fluorescence and Confocal Fluorescence Microscopy

Bulk-cell GFP fluorescence measurements were made in a Beckman Coulter Paradigm plate reader with excitation/emission wavelengths of 485/535 nm. Confocal Fluorescence Microscopy was performed with cells at an OD (600 nm) of 0.8 – 1.2, using a 100x 1.4 NA (numerical aperture) oil immersion objective on a Leica SD6000 microscope attached to a Yokogawa CSU-X1 spinning disc head with a 488 nm laser and controlled by Metamorph software. Z series were recorded with a 200 nm step size and analyzed using ImageJ.

[^{3}H]-Cellobiose Transport Assays and Kinetic Parameters

Transport assays were performed using a modification of the oil-stop method (Arendt, Ri, Yates, & Ullman, 2007). Yeast strains expressing either *cdt-1* or *cdt-2* fused to GFP were grown to an OD (600 nm) of 1.5 – 3.0 in selective media, washed 3x with assay buffer (30 mM MES-NaOH [pH 5.6] and 50 mM ethanol), and resuspended to an OD (600 nm) of 20. To start transport reactions, 50 µL of cells were added to 50 µL of [^{3}H]-cellobiose layered over 100 µL of silicone oil (Sigma 85419). Reactions were stopped by spinning cells through oil for 1 minute at 17,000 g, 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%. Kinetic parameters were determined by measuring the linear rate of [^{3}H]-cellobiose uptake over 3 minutes for cellobiose concentrations between 0.5 and 200 µM. 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®. V_{max} values were normalized for differences in transporter abundance by measuring the GFP fluorescence from 100 µL of cells at OD (600 nm) 20 immediately before beginning transport assays. Kinetic parameters reported in the text are the mean ± SEM from three separate experiments. Competition assays were performed by measuring transport of 50 µM [^{3}H]-cellobiose over 20 s in the presence of 250 µM of the respective competitors.

[^{3}H]-Cellobiose Transport Assays in the presence of carbonyl cyanide m-chlorophenyl hydrazone (CCCP)

To determine the sensitivity of cellobiose transport by CDT-1 or CDT-2 to the strength of the plasma membrane proton gradient, strains were prepared as in a standard [^{3}H]-cellobiose transport assay described above, then split into 350 µL aliquots. These were held on ice. To assay the transport rate at a given concentration of CCCP, 0.35 µL of a 1000x CCCP stock (made in 100% ethanol) was added to a single 350 µL aliquot of cells. This aliquot was then incubated for 10 minutes in a room-temperature water bath. After this incubation, a transport assay was performed exactly as outlined above, except that the 50 µL of buffered [^{3}H]-cellobiose that was layered above the silicone oil also contained the appropriate concentration of CCCP. This process was repeated sequentially for each tested

concentration of CCCP. Controls with a CCCP concentration of 0 μM were incubated for 10 minutes with 0.35 μL of 100% ethanol.

Measurement of β-glucosidase activity in culture media

To assay for contaminating β-glucosidase activity, strains were grown in 5 mL of complete minimal media with appropriate dropouts, overnight. For large scale experiments, these starter cultures were washed 3x with 25 mL ddH_2O and resuspended to an OD (600 nm) of 0.1 in 50 mL of Yeast Nitrogen Base (YNB) plus the appropriate Complete Supplemental Media (CSM) and 2% (w/v) cellobiose in 250 mL shaker flasks. Cultures were grown at 30 °C for 84 hours, at which point the OD (600 nm) was determined, and the cell-free culture supernatant collected by filtration through a 0.2 μm filter. For small-scale experiments, starter cultures were washed as above, and resuspended to an O.D. (600 nm) of 0.1 in 400 μL of YNB plus the appropriate CSM and 0.5% (w/v) of either cellotriose or cellotetraose. Cultures were grown at 30 °C for 96 hours in a Bioscreen C™ at which point the OD (600 nm) was determined, and the cell-free culture supernatant collected as above. Filtrates were colorimetrically assayed for β-glucosidase activity by monitoring the release of *para*-Nitrophenol (pNP) from *para*-Nitrophenyl β-D-glucopyranoside (pNPG) (Wood & Bhat, 1988). Briefly, 80 μL of the filtrate was mixed with 20 μL of 5 mM pNPG and incubated for 1 hour at 30 °C. At this point, 133 μL of 0.4 M glycine-NaOH [pH 10.8] was added, and the absorbance at 405 nm determined in a Beckman Coulter Paradigm plate reader. Purified NCU00130 (GH1-1, **Figure 2-10**) was used as a control.

Large-scale Yeast Growth

To monitor growth on different carbon sources, engineered strains were grown in 5 mL of complete minimal media with appropriate dropouts, overnight. These starter cultures were washed 3x with 25 mL of ddH_2O, and resuspended to an OD (600 nm) of 0.1 in 50 mL YNB plus the appropriate CSM and 2% (w/v) cellobiose. Cultures were grown in 250 mL unbaffled2 flasks at 30 °C, with shaking at 200 RPM. The change in OD (600 nm) was monitored by periodically removing samples.

3. Functional analysis of CDT-1, CDT-2 and their Orthologs in other Fungi

Partially taken from: Glass, N. L., Tian C., Beeson W. T., Cate J. H., Galazka, J. M., Ha S. J., Jin Y. S., Kim S. R., Li S., Yang X. (2010). US Patent Application 201110020910. Methods and compositions for improving sugar transport, mixed sugar fermentation and production.

3.1 Abstract

To identify functionally important residues within the cellodextrin transporters, CDT-1 and CDT-2, conserved amino acids were mutated to alanine. The functional consequences of these mutations were determined using growth assays in *S. cerevisiae*, revealing mutations that both increased and decreased the cellobiose transport rate of CDT-1 and CDT-2. In addition, the ability of CDT-1, CDT-2 and NCU05853 orthologs from diverse fungi to transport cellobiose was determined using an identical growth assay in *S. cerevisiae*. This identified a number of functional cellobiose transporters.

3.2 Results and Discussion

The open reading frame of CDT-1, CDT-2 and NCU05853 from *N. crassa* was used as a BLAST query against the genome of fungi known to degrade various parts of lignocellulose. The top hit from this query was then used as a blast query against the *N. crassa* genome. If this second query matched the original input sequence (i.e. the top hit in a given fungi using CDT-1 yields CDT-1 as a top hit in *N. crassa*) it was considered the ortholog of the *N. crassa* gene. A multiple sequence alignment was then performed using MUSCLE, and this alignment was manually inspected to identify conserved amino acids. Amino acids were identified that were conserved in: (i) all major facilitator superfamily sugar transporter; (ii) both the CDT-1 and the CDT-2 clades; (iii) only the CDT-1 or CDT-2 clades. This process was used to identify 96 residues within CDT-1 and CDT-2 that fell into one of these three categories. These were mutated to alanine by the Quickchange® protocol. The functional consequences of each of these mutations were determined using a growth assay in *S. cerevisiae*. A *S. cerevisiae* expressing the intracellular β-glucosidase, *gh1-1*, was transformed with each alanine mutant individually. The growth rate of these strains with cellobiose as a sole carbon source was then determined (**Figure 3-1, Table 3-2, Figure 3-3, Table 3-4**).

A spectrum of effects was seen from these mutations. In both CDT-1 and CDT-2, mutations resulted in growth rates that were either approximately identical to, greater than, or less than the growth rates of strains with WT CDT-1 or CDT-2. For example, in CDT-1, the G91A and F335A mutations lead to growth rates that were 131% and 118% of WT, while the R174A and E194A mutations lead to growth rates that were 13% and 12% of WT. Likewise, in CDT-2, the Q207A and D496A mutations lead to growth rates that were

217% and 136% of WT, while the S224A and Y421A mutation lead to growth rates that were 32% and 25% of WT.

A subset of the identified CDT-1, CDT-2 and NCU05853 orthologs from other fungal species were tested for their ability to transport cellobiose. Thirteen orthologs of CDT-1, five of CDT-2 and four of NCU05853 were selected (**Table 3-5**). An unrooted phylogenetic tree of these transporters shows that the CDT-2 and NCU05853 orthologs cluster closely with themselves (**Figure 3-6**). While the CDT-1 orthologs group with themselves as well, there is considerably more diversity within this set.

To test if the selected transporters imported cellobiose, the genes were codon-optimized for expression in *S. cerevisiae*, synthesized, and cloned into a 2μ plasmid under the control of the yeast PGK1 promoter by Genescript. These constructs were transformed individually into a *S. cerevisiae* containing the intracellular β-glucosidase, GH1-1. The ability of these strains to grow with cellobiose as a sole carbon source was then determined **(Figure 3-7**). As compared to WT CDT-1, most strains grew slowly on cellobiose. However, a few strains did grow at reasonable rates. In particular, strains containing the *Chaetomium globosum* and *Aspergillus clavatus* orthologs of CDT-1 grew with rates that were above those of a control containing the intracellular β-glucosidase (GH1-1) but without a transporter.

3.3 Materials and Methods

Mutagenesis of CDT-1 and CDT-2 and strain construction

Alanine mutants were produced in previously described 2μ yeast expression plasmids (Galazka et al., 2010). These plasmids contained the CDT-1 or CDT-2 open reading frame fused to GFP, and the *S. cerevisiae* PGK1 promoter and CYC transcriptional terminator within the pRS426 backbone. The QuickChange® protocol was used to introduce alanine mutations, and results were confirmed by sequencing the mutated plasmids. CDT-1 and CDT-2 orthologs were codon-optimized, synthesized and cloned by Genescript. Orthologs were cloned into a previously described (Galazka, et al., 2010) 2μ yeast expression plasmid (pRS426) that contained the *S. cerevisiae* PGK1 promoter and CYC transcriptional terminator. All plasmids were transformed into the *S. cerevisiae* strain YPH499 (*MAT*a *ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*), which contained a previously described (Galazka, et al., 2010) plasmid bearing the GH1-1 open reading frame and the *S. cerevisiae* PGK1 promoter within the pRS425 backbone.

Determination of strain growth rates

Colonies corresponding to independent transformants of *S. cerevisiae* strain YPH499 containing GH1-1 and a given alanine mutant of CDT-1 or CDT-2 or of a given ortholog of CDT-1 or CDT-2 were resuspended in 400 μL of YNB (with ammonium sulfate) plus 2% cellobiose and CSM (–uracil, –leucine). This gave a starting OD (600 nm) of ~0.2. Growth curves were recorded using a Bioscreen C™, and growth rates determined from the exponential phase of the curve.

4. *Saccharomyces cerevisiae* Engineered to Co-ferment Cellobiose and Xylose

Partially taken from: Ha, S. J., Galazka, J. M., Rin Kim, S., Choi, J. H., Yang, X., Seo, J. H., et al. (2011). Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proc Natl Acad Sci U S A*, *108*(2), 504-509.

4.1 Abstract

The use of plant biomass for biofuel production will require efficient utilization of the sugars in lignocellulose, primarily glucose and xylose. However, strains of *Saccharomyces cerevisiae* presently used in bioethanol production ferment glucose but not xylose. Yeasts engineered to ferment xylose cannot utilize xylose until glucose is completely consumed. To overcome this bottleneck, we engineered *S. cerevisiae* to co-ferment mixtures of xylose and cellobiose. In these strains, hydrolysis of cellobiose takes place inside the yeast cell through the action of an intracellular β-glucosidase following import by a cellodextrin transporter. Intracellular hydrolysis of cellobiose minimizes glucose repression of xylose fermentation, allowing co-consumption of cellobiose and xylose. The resulting strain co-fermented cellobiose and xylose simultaneously and exhibited improved ethanol yield when compared to fermentations with either cellobiose or xylose as sole carbon sources. We also observed improved yields and productivities from co-fermentation experiments performed with simulated lignocellulosic hydrolyzates, suggesting this is a promising strategy for lignocellulosic biofuel production.

4.2 Introduction

There is a long history of microbial fermentation of glucose derived from cornstarch or sugarcane to fuels and chemicals. Modifying this process to ferment the sugars produced from the depolymerization of plant cell walls is necessary for lignocellulosic biofuel production (Wyman, 2007). While hydrolyzates from cornstarch and sugarcane contain only hexoses, hydrolyzates from lignocellulosic biomass contain various hexoses and pentoses. Hexoses are produced following the hydrolysis of cellulose by fungal cellulases, which primarily generate cellobiose that can be further hydrolyzed to glucose by β-glucosidases. Pentoses are produced by acid treatment of hemicellulose, which releases xylose and arabinose. While the composition is variable, plant biomass hydrolyzates consists of ~70% cellodextrins and glucose, and ~30% xylose (Carroll & Somerville, 2009). Successful conversion of lignocellulosic biomass into biofuel will therefore require organisms capable of efficient utilization of xylose as well as cellodextrins and glucose (Ragauskas et al., 2006; Stephanopoulos, 2007).

The traditional glucose-fermenting yeast, *Saccharomyces cerevisiae*, cannot ferment xylose because it does not possess a functional xylose-assimilation pathway. However, as wild-type *S. cerevisiae* can metabolize xylulose using the pentose phosphate pathway, the introduction of genes (*XYL1* and *XYL2*) encoding xylose reductase (XR) and xylitol

dehydrogenase (XDH) from *Pichia stipitis* (*Scheffersomyces stipitis*) facilitates xylose assimilation in this yeast (Kötter & Ciriacy, 1993; Tantirungkij, Nakashima, Seki, & Yoshida, 1993). In addition, either overexpression of *XKS1* coding for endogenous xylulokinase (Ho, Chen, & Brainard, 1998; Toivari, Aristidou, Ruohonen, & Penttila, 2001), or introduction of *XYL3* coding for *P. stipitis* xylulokinase (Jin, Ni, Laplaza, & Jeffries, 2003), significantly improved the rate and yield of xylose fermentation in *S. cerevisiae*. However, reported ethanol yields and productivities from xylose fermentation by these engineered strains were lower than those from xylose fermentation by *P. stipitis*, as well as those from glucose fermentation by *S. cerevisiae* (Hahn-Hagerdal, Karhumaa, Jeppsson, & Gorwa-Grauslund, 2007; T. W. Jeffries & Jin, 2004). Moreover, both natural and engineered microorganisms showed reduced ethanol tolerance during xylose fermentation as compared to glucose fermentation (T. W. Jeffries & Jin, 2000).

Combined with the lower fermentation rate, the reduced ethanol tolerance during xylose fermentation poses a significant problem for the fermentation of lignocellulosic hydrolyzates that contain high concentrations of glucose (70–100 g/L) and xylose (40–60 g/L). This is because microorganisms utilize glucose preferentially. Therefore, xylose fermentation only begins once glucose is depleted and significant quantities of ethanol are present. This ethanol further reduces the xylose fermentation rate. As a result, sequential utilization of xylose and glucose is a significant barrier to the successful utilization of the mixed sugars in lignocellulosic hydrolyzates.

To bypass the problems caused by glucose repression in a mixed sugar fermentation, we developed a unique strategy to co-ferment cellobiose (a disaccharide of glucose) with xylose simultaneously (**Figure 4-1**). Wild-type *S. cerevisiae* cannot assimilate cellobiose because it lacks both a cellobiose transporter and a β-glucosidase capable of hydrolyzing cellobiose into glucose. Therefore, we introduced a newly discovered cellodextrin transporter and intracellular β-glucosidase from the cellulolytic fungi, *Neurospora crassa* (Galazka, et al., 2010), into *S. cerevisiae* strains engineered to ferment xylose. A different approach, which enables co-fermentation of cellobiose and xylose through a display of β-glucosidase on the surface of xylose-fermenting *S. cerevisiae*, has been reported (Nakamura et al., 2008). Although both approaches target co-fermentation of cellobiose and xylose, the two differ in the location of cellobiose hydrolysis and its effect on xylose transport. The surface display of β-glucosidase generates glucose extracellularly. In contrast, in the present design cellobiose is hydrolyzed intracellularly following transport. Because xylose uptake by *S. cerevisiae* is facilitated by hexose transporters which are severely inhibited by glucose (Jojima, Omumasaba, Inui, & Yukawa, 2010; W. J. Lee, Kim, Ryu, Bisson, & Seo, 2002; Runquist, Hahn-Hagerdal, & Radstrom, 2010), the extracellular glucose generated by surface display of β-glucosidase can inhibit xylose uptake (Nakamura, et al., 2008) whereas the intracellular glucose from intracellular hydrolysis of cellobiose will not. Perhaps due to this fundamental distinction, our strategy achieves faster xylose and cellobiose co-consumption rates, and may prove optimal for biofuel production from lignocellulosic feedstocks.

4.3 Results and Discussion

The *N. crassa* cellodextrin transporter, *cdt-1*, was introduced into a *S. cerevisiae* strain expressing an intracellular β-glucosidase (*gh1-1*) to form the strain, D801-130. This strain consumed 40 g/L of cellobiose within 24 h, producing 16.8 g/L of ethanol (**Figure 4-2**). The volumetric productivity of cellobiose fermentation ($P_{\text{Ethanol/Cellobiose}}$ = 0.7 g/L·h) was lower than that of glucose fermentation ($P_{\text{Ethanol/Glucose}}$ = 1.2 g/L·h), and ethanol yield from cellobiose ($Y_{\text{Ethanol/Cellobiose}}$ = 0.42 g/g or 0.26 mol/mol C) was slightly lower than ethanol yield from glucose ($Y_{\text{Ethanol/Glucose}}$ = 0.43 g/g or 0.28 mol/mol C) under the same culture conditions. However, the observed cellobiose consumption rate and ethanol yield by the D801-130 were an improvement over *S. cerevisiae* strains engineered to ferment cellobiose through surface display of β-glucosidase (Kotaka et al., 2008a; Nakamura, et al., 2008). These results suggest that simultaneous expression of *cdt-1* and *gh1-1* in *S. cerevisiae* results in an efficient cellobiose-fermenting yeast.

Attempts to engineer xylose fermentation into *S. cerevisiae* have suffered from low yields due to xylitol accumulation, and low productivities due to slow xylose fermentation rates (Hahn-Hagerdal, Karhumaa, Jeppsson, et al., 2007; T. W. Jeffries & Jin, 2004). In order to improve the yield and productivity of xylose fermentation, we undertook both rational and combinatorial approaches. First, we drastically reduced xylitol accumulation by reconstituting an efficient xylose metabolic pathway. XR prefers NADPH to NADH while XDH solely uses NAD^+ as a cofactor (Rizzi, Harwart, Buithanh, & Dellweg, 1989; Verduyn et al., 1985). This results in a redox imbalance that is thought to result in xylitol accumulation. This imbalance has been corrected by replacing wild-type XR with various mutant XRs that exhibit higher preferences for NADH (Bengtsson, Hahn-Hagerdal, & Gorwa-Grauslund, 2009; Watanabe et al., 2007). However, these efforts resulted in much slower xylose assimilation rates despite reducing the amounts of xylitol produced, as the mutant XRs had reduced specific activities as compared to wild-type XR.

In contrast to previous studies that relied on either wild-type XR or a mutant XR, we expressed both wild-type XR and a mutant XR (R276H) in *S. cerevisiae*, along with XDH and XK to construct a functional xylose metabolic pathway in *S. cerevisiae*. The XR mutant (R276H) had been reported to exhibit much higher preference for NADH whereas wild-type XR showed two-fold higher preference for NADPH (Watanabe, et al., 2007). The resulting xylose-fermenting *S. cerevisiae* strain (DA24) expressing both wild-type XR and mutant XR (R276H) exhibited comparable XR activities with both NADPH and NADH in an *in vitro* XR activity assay. Unlike other engineered strains showing higher XR activities with preferred cofactors, strain DA24 showed similar XR activities regardless of cofactors (**Table 4-3**).

Second, we further improved the xylose-fermentation rate of DA24 using an evolutionary engineering approach (Sauer, 2001). The DA24 strain rapidly consumed xylose and produced ethanol with consistent ethanol yields ($Y_{\text{Ethanol/Xylose}}$ = 0.31–0.32 g/g or 0.21–0.22 mol/mol C) in both shaker-flask and bioreactor fermentation experiments (Fig. S2). The DA24 strain also produced negligible amounts of xylitol. Still, the DA24 strain consumed xylose slower than the naturally existing xylose-fermenting yeast, *P. stipitis*.

Evolutionary engineering was used to isolated the strain, DA24-16, by repeated subculturing of DA24 on xylose-containing medium. The selected strain (DA24-16) showed much faster xylose fermentation rates when compared to the parental strain under various culture conditions (**Table 4-4**). The specific xylose uptake rates, ethanol yields and ethanol production rates of the DA24 and DA24-16 strains were much higher than engineered *S. cerevisiae* strains reported previously. Remarkably, the DA24-16 strain consumed xylose almost as fast as *P. stipitis*, the fastest xylose-fermenting yeast known. However, the ethanol yield from DA24-16 was slightly lower than that from *P. stipitis* (**Figure 4-5**).

To further improve this newly engineered xylose-fermenting strain, we introduced genes coding for a cellodextrin transporter and a β-glucosidase (*cdt-1* and *gh1-1*) to construct a strain capable of consuming cellobiose and xylose simultaneously. We hypothesized that glucose repression of xylose utilization may be alleviated in this strain, due to the intracellular hydrolysis of cellobiose. Indeed, a strain with *cdt-1* integrated into the genome of DA24-16 and expressing *gh1-1* from a multicopy plasmid (strain DA24-16-BT3) co-consumed cellobiose and xylose and produced ethanol with yields of 0.38–0.39 g/g in all conditions (**Table 4-6**).

We also investigated potential synergism during co-fermentation by culturing DA2416-BT3 under three different conditions: 40 g/L of cellobiose, 40 g/L of xylose, and 40 g/L of both sugars (total 80 g/L of sugar). Interestingly, DA24-16BT3 was able to co-consume 80 g/L of a cellobiose/xylose mixture within the same period that was required to consume 40 g/L of cellobiose or 40 g/L xylose separately (**Figure 4-7**). Moreover, DA24-16BT3 produced ethanol with a higher yield (0.39 g/g or 0.24 mol/mol C) from a mixture of cellobiose and xylose as compared to ethanol yields (0.31–0.33g/g or 0.21–0.22 mol/mol C) from single sugar fermentations (cellobiose or xylose). Ethanol productivity also dramatically increased from 0.27 g/L·h to 0.65 g/L·h during co-fermentation. These results suggest that co-fermentation of cellobiose and xylose can enhance overall ethanol yield and productivity.

We also performed fermentation experiments comparing our engineered *S. cerevisiae* strain (DA24-16BT3) to *P. stipitis*, a yeast capable of co-fermenting cellobiose and xylose efficiently. Using a simulated hydrolyzate (10 g/L of glucose, 80 g/L of cellobiose, and 40 g/L of xylose) based on the composition of energycane, the DA24-16BT3 consumed glucose first, and co-consumed cellobiose and xylose rapidly. A total of 130 g/L of sugars were consumed within 60 h even though small inoculums were used (OD = 1.3, or ~0.4 g dried cell/L). In contrast, *P. stipitis* could not finish fermenting the sugar mixture within the same period under identical culture conditions (**Figure 4-8**). DA24-16BT3 produced 48 g/L of ethanol within 60 h ($Y_{\text{Ethanol/Sugars}}$ = 0.37 g/g or 0.23 mol/mol C, and $P_{\text{Ethanol/Sugars}}$ = 0.79 g/L·h). Fermentation experiments using a bioreactor with the same or higher inoculums yielded consistent results. We also performed fermentation experiments of sugar mixtures containing extreme concentrations of glucose and xylose (100 g/L of glucose and 60 g/L of xylose) and cellobiose and xylose (100 g/L of cellobiose and 60 g/L xylose). As illustrated in **Figure 4-1**, xylose fermentation was severely delayed when the mixture of glucose and xylose was used. However, while co-fermentation of cellobiose and

xylose showed slower ethanol production initially, it ultimately resulted in higher ethanol production (**Figure 4-9**).

Here we demonstrate a unique strategy to enable the co-fermentation of glucose and xylose by *S. cerevisiae*. By combining an efficient xylose utilization pathway with a cellobiose transport system, we bypassed problems caused by glucose repression. As a result, the engineered yeast co-fermented two non-metabolizable sugars in cellulosic hydrolyzates into ethanol synergistically. This method improves lignocellulosic biofuel production in two ways. First, most fungal cellulase cocktails require that additional β-glucosidase be added to fully convert cellobiose into glucose for subsequent fermentation by *S. cerevisiae*. Cellobiose and xylose co-fermentation enables the use of cellulase cocktails with limited β-glucosidase activity, reducing the enzyme requirements, and ultimately the costs of the cellulose saccharification process. Second, the synergy of cellobiose and xylose co-fermentation significantly increases ethanol productivity from these sugars, potentially improving fermentation economics.

Although we obtained promising results from our co-fermentation experiments, further improvements to these strains can likely be made. For example, we observed transient accumulation of cellodextrins in the medium during cellobiose fermentation. It is likely that these were generated by the transglycosylation activity (Christakopoulos, Kekos, Macris, Goodenough, & Bhat, 1994) of the intracellular β-glucosidase, and secreted by the cellodextrin transporter, which could facilitate the transport of cellodextrins in both directions (intracellular ↔ extracellular). The transient cellodextrin accumulation may not reduce product yields because our engineered yeast consumes the cellodextrins after cellobiose depletion. However, the accumulated cellodextrins might decrease productivity because the transport or hydrolysis rates of cellotriose and cellotetraose might be slower than that of cellobiose.

We also observed that small amounts of glucose were constantly present in the medium during the co-fermentation of cellobiose and xylose. Because even small amounts of glucose can repress xylose fermentation, glucose accumulation must be limited. We hypothesize that the relative expression levels of the cellodextrin transporter and β-glucosidase control the glucose accumulation rate. This is supported by the observation that more glucose is accumulated in the medium when *cdt-1* is introduced on a multicopy plasmid than when *cdt-1* is integrated into the genome. Future optimization of the expression levels of the cellodextrin transporter and β-glucosidase, or the identification of β-glucosidases with reduced transglycosylation activities, may reduce the accumulation of glucose and cellodextrins during co-fermentation.

In this study, we measured the capacity of our engineered strain to ferment various mixtures of sugars meant to mimic hydrolyzates from plant biomass. However, we predict that the capacity of our strain to co-ferment cellodextrins and xylose will make it particularly useful during the Simultaneous Saccharification and Co-Fermentation (SSCF) of pretreated plant biomass (Doran-Peterson, et al., 2009). During SSCF, hemicellulose would first be hydrolyzed by acid pretreatment, resulting in xylose and cellulose. Then, fungal cellulases and the yeast strain developed here would be added, allowing the co-

conversion of xylose and cellobiose into ethanol. Because little extracellular glucose is produced in this scheme, repression of xylose utilization would be minimized, and co-fermentation would proceed rapidly and synergistically.

Although the *S. cerevisiae* strain used in this study was a laboratory strain, the fermentation performance of the engineered strain was very impressive as compared to published results (Hahn-Hagerdal, Karhumaa, Fonseca, Spencer-Martins, & Gorwa-Grauslund, 2007). We envision that key fermentation parameters (yield and productivity) can be improved further if we employ industrial strains as a platform. Finally, applications of our co-fermentation strategy would not be limited to ethanol production. Because it is a foundational technology, the strategy presented here can be combined with other genetic manipulations of *S. cerevisiae* to produce commodity chemicals or advanced biofuels.

4.4 Materials and Methods

Strains and Plasmid Construction

S. cerevisiae D452-2 (*MATalpha, leu2, his3, ura3, and can1*) was used for engineering of xylose and cellobiose metabolism in yeast. *Escherichia coli* DH5 (*F–recA1 endA1 hsdR17 [rK– mK+] supE44 thi-1 gyrA relA1*) (Invitrogen) was used for gene cloning and manipulation. *P. stipitis* CBS 6054 (T. W. Jeffries et al., 2007) was obtained from Thomas Jeffries at University of Wisconsin-Madison. Strains and plasmids used in this work are described in **Table 4-10**. The primers used for confirming the transformation of expression cassettes containing *cdt-1*, *cdt-2*, and *gh1-1* are listed in **Table 4-11**.

Medium and Culture Conditions

E. coli was grown in Luria-Bertani medium; 50 μg/mL of ampicillin was added to the medium when required. Yeast strains were routinely cultivated at 30 °C in YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) with 20 g/L glucose. 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 (Bio 101), which supplied appropriate nucleotides and amino acids.

Fermentation Experiments

Yeast cultures were grown in YP medium containing 20 g/L of glucose or 20 g/L of cellobiose to prepare inoculums for xylose or cellobiose fermentation experiments, respectively. Cells at mid-exponential phase from YP media containing 20 g/L of glucose or cellobiose were harvested and inoculated after washing twice by sterilized water. Flask fermentation experiments were performed using 50 mL of YP medium containing appropriate amounts of sugars in 250 mL flask at 30 °C with initial O.D. (600 nm) of ~1 or ~10 under oxygen limited conditions. All of the flask fermentation experiments were repeated independently. The variations between independent fermentations were less than 5%. Fermentation profiles shown in figures are from a representative fermentation. Bioreactor fermentations were performed in 400 mL of YP medium containing appropriate amounts of sugars using Sixfors Bioreactors (Appropriate Technical Resources, Inc) at 30

°C with an agitation speed of 200 rpm under oxygen limited conditions. Initial cell densities were adjusted to an O.D. (600 nm) of ~1 or ~10.

5. Rapid fermentation of cellobiose *via* phosphorolysis by an engineered *S. cerevisiae* requires a thermodynamic "push" and "pull"

5.1 Abstract

The yeast *Saccharomyces cerevisiae* can be engineered to ferment cellobiose to ethanol using either a hydrolytic or phosphorolytic pathway. Strains with a cellobiose transporter and an intracellular cellobiose phosphorylase initially fermented cellobiose slowly, approximately one-third the rate of strains containing a hydrolytic pathway consisting of the same cellobiose transporter and an intracellular cellobiose hydrolase. Directed evolution produced a phosphorolytic strain with approximately three-fold faster fermentation rates. Remarkably, this improvement was due solely to a point mutation in the cellobiose transporter that raised its transport rate. Other transporter mutants with elevated transport rates also improved fermentation rates in phosphorolytic strains. The cellobiose fermentation rate of phosphorolytic strains was also improved by the over-expression of hexokinase. These observations suggest that the phosphorolytic strain is limited by the energetics of cellobiose phosphorolysis (ΔG^o = +3.6 kJ mol^{-1}), and that a thermodynamic "push" and "pull" from the reactions immediately upstream (transport) and downstream (phosphorylation of glucose) are necessary to achieve high fermentation rates.

5.2 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 & Somerville, 2009). They are abundant in agricultural and municipal wastes, and in dedicated energy crops (Somerville, Youngs, Taylor, Davis, & Long, 2010). The yeast, *Saccharomyces cerevisiae*, is a favored platform for these engineering efforts because it is robust, simple to manipulate genetically, and capable of facilitating high carbon fluxes to central metabolic pathways (F. Zhang, Rodriguez, & Keasling, 2011). Despite this *S. cerevisiae* has a number of drawbacks including an inability to naturally ferment pentose sugars (Hahn-Hagerdal, Karhumaa, Fonseca, et al., 2007), sensitivity to solvents (Ma & Liu, 2010), and sensitivity to inhibitory compounds found in deconstructed plant materials (Almeida, Runquist, Sanchez i Nogue, Liden, & Gorwa-Grauslund, 2011). These drawbacks have been addressed to varying degrees (Hahn-Hagerdal, Karhumaa, Fonseca, et al., 2007; Nevoigt, 2008; Ostergaard, Olsson, & Nielsen, 2000).

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 (Y. H. Zhang & Lynd, 2004). To allow cellobiose consumption, *S. cerevisiae* has been modified to either: (i) secrete or surface-

display a β-glucosidase to hydrolyze cellobiose to glucose extracellularly (Machida, Ohtsuki, Fukui, & Yamashita, 1988; Saitoh, et al., 2008); (ii) import cellobiose with a cellobiose transporter for intracellular hydrolysis by a β-glucosidase (Galazka, et al., 2010; Ha et al., 2011; Li et al., 2010); (iii) import cellobiose with a lactose permease for intracellular phosphorolysis by a cellobiose phosphorylase (Sadie, Rose, den Haan, & van Zyl, 2011). In the two hydrolytic pathways, the o-glycosidic linkage of cellobiose is cleaved by a hydrolase (EC 3.2.1.21) with H_2O to produce glucose, while in the phosphorolytic pathway it is cleaved by a phosphorylase (EC 2.4.1.20) with inorganic phosphate (P_i) to produce glucose and α-glucose-1-phosphate (Gluc-1P). The difference between the hydrolytic and 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 (**Figure 5-1**).

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 cellobiose phosphorolysis reaction (ΔG^o = +3.6 kJ mol^{-1}), rapid substrate supply and product removal may be necessary. Here, we describe an engineered *S. cerevisiae* that rapidly ferments cellobiose to ethanol *via* intracellular cellobiose phosphorolysis. Furthermore we show that its cellobiose fermentation rate can be improved by a thermodynamic "push" and "pull". We directly compare its performance to a strain reliant on cellobiose hydrolysis, and describe the genetic manipulations necessary to improve the fermentation rates of engineered strains with the phosporolytic pathway.

5.3 Results and Discussion

Cellobiose phosphorylase genes from *Saccharophagus degradans* (SdCBP), *Celvibrio gilvus* (CgCBP), and *Clostridium thermocellum* (CtCBP) were codon optimized, synthesized and cloned into 2μ plasmids. These plasmids were transformed, along with a plasmid carrying a cellodextrin transporter gene (CDT-1) tagged with green fluorescent protein (GFP) into *S. cerevisiae* strain D452-2. All three resulting strains fermented cellobiose into ethanol (**Figure 5-2**). The cellobiose consumption rates of the strains expressing CgCBP, SdCBP, or CtCBP were similar (0.61~0.69 g/L-h), as were the ethanol production rates of these strains (0.22~0.26 g/L-h). After 108 hours, almost all cellobiose was fermented to ethanol with a yield of 0.37~0.38 g/g, and there was no appreciable accumulation of acetate, glucose or glycerol. 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.

CBP phosphorolytically cleaves cellobiose to glucose and α-glucose-1-phosphate (Gluc-1P). Gluc-1P must be converted to glucose-6-phosphate (Gluc-6P) by phosphoglucomutase (EC 5.4.2.2) to enter the glycolytic pathway. In *S. cerevisiae*, *PGM2* is transcriptionally downregulated during glycolytic growth (Oh & Hopper, 1990). Therefore a set of strains in which *PGM2* was expressed ectopically along with *cdt-1* and one CBP was tested. However, these strains showed slightly reduced performance as compared to those without ectopic *PGM2*.

An evolutionary engineering approach was used 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. To identify mutations responsible for this improvement, both 2μ plasmids (pRS425-SdCBP and pRS426-CDT-1-GFP) were isolated from the evolved strain and sequenced. This 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 completely explains the improved performance, as retransforming the isolated plasmid into un-evolved *S. cerevisiae* recapitulated the result (**Figure 5-3 A**). Strains with CDT-1(F213L) and SdCBP consumed cellobiose and produced ethanol with rates of 1.72 ± 0.11 g/L-h and 0.74 ± 0.05 g/L-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 to 0.44 g/g, and there was no detectable accumulation of acetate, glucose, glycerol, or cellodextrins.

Strains with CDT-1 an intracellular cellobiose hydrolase 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 *S. cerevisiae* along with the intracellular cellobiose hydrolase, *gh1-1*. Only a slight improvement in the cellobiose fermentation parameters was seen compared to a strain with WT CDT-1 and GH1-1 (**Figure 5-3 B**). 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).

To determine if other mutants of CDT-1 would have a similar effect on cellobiose fermentation, 7 additional CDT-1 mutants were constructed and tested. These all replaced one amino acid with an alanine (G91A, F335A, Q104A, F170A, E194A, R174A). Cellobiose consumption rates of strains with these transporters and GH1-1 varied, with G91A > F335A > F213L > WT > F170A > Q104A > R174A > E194A (**Figure 5-4 and Table 5-5**). Ethanol production rates followed this same trend. However, a different trend was

observed in strains expressing one mutant transporter and SdCBP. In this case, F213L > G91A > WT > Q104A > F170A > F335A > R174A > E194A. The most notable difference being that the optimal transporter was different for strains with GH1-1 or SdCBP.

To assess transporter function directly, a Michaelis-Menten analysis of the 4 transporters giving the best fermentation rates (WT, G91A, F335A, F213L) was performed by measuring the rate of [^{3}H]-cellobiose uptake into cells at various concentrations of [^{3}H] cellobiose (**Figure 5-6 and Table 5-7**). Compared to WT (K_m = 7.6 ± 1.5 μM, V_{max} = 0.60 ± 0.03 pmol/s), all of the mutants had a lower affinity for cellobiose but a higher V_{max}. F213L, F335A and G91A had a K_m of 188.8 ± 55.3 μM, 114.8 ± 46.4 μM and 43.5 ± 10.1 μM, respectively. These same transporters had a V_{max} of 2.38 ± 0.31 pmol/s, 1.75 ± 0.26 pmol/s and 1.87 ± 0.12 pmol/s, respectively. Thus, transporters that allow faster fermentation rates also transport cellobiose at a higher maximum velocity.

The distinct differences in strain performance when cellobiose is cleaved by hydrolysis *versus* phosphorolysis despite the use of identical transporter genes, could suggest 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. The extracts were prepared from cells during the exponential phase of cellobiose fermentation by strains with CDT-1 and either GH1-1 or SdCBP. The strains expressed *cdt-1* at identical levels according to the amount of GFP fluorescence present per cell (**Figure 5-8**). For additional comparisons, extracts were prepared from WT D452-2 during the exponential phase of glucose fermentation.

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 extract (as determined by the Bradford assay) was measured (**Figure 5-8**). Cellobiase activity was defined as the rate of glucose produced from cellobiose regardless of the mechanism, and was similar in strains with GH1-1 or SdCBP. As expected, there was no detectable cellobiase activity in extracts of WT D452-2. It is important to note that this assay underestimates the activity of CBP by 50% because the Gluc-1P produced by this enzyme is not accounted for. 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 (**Figure 5-8**).

As noted, during cellobiose fermentations performed with strains with CDT-1 and CDT-1 mutants 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 (M. 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 cell extracts by IMAC. The

enriched enzymes were incubated with 20% (w/v) cellobiose for 24 hours, and the reaction products were analyzed by HPLC. GH1-1 but not SdCBP had significant transglycosylation activity under these conditions, producing 0.44 mg cellotriose and 0.09 mg cellotetraose from 2 mg cellobiose (**Figure 5-9**). The kinetic parameters of the enriched proteins were also measured. GH1-1 has a K_M for cellobiose of 320 ± 0.045 μM and a k_{cat} of ~7 s^{-1}, while SdCBP has a K_M for cellobiose of 647 ± 0.058 μM and a k_{cat} of ~7 s^{-1} (**Figure 5-10 and Table 5-11**).

Cellobiose is not naturally found in the cytosol of *S. cerevisiae*, and its presence could have numerous unforeseen consequences. Hexokinase (EC 2.7.1.1) binds tightly to glucose (K_m = 130 μM (Gao & Leary, 2003)), and therefore could non-productively interact with intracellular cellobiose in these engineered strains. To test the inhibitory properties of cellobiose on the two hexokinases and one glucokinase from *S. cerevisiae*, they were expressed and purified from *E. coli*. The activity of the pure enzymes was then assayed in the presence and absence of up to 184 mM cellobiose. At these extreme concentrations of cellobiose, Hxk1p was unaffected, while the activity of Hxk2p and Glk1p were reduced by ~20% (**Figure 5-12**).

To determine if hexokinase activity was limiting in the engineered pathways, the same two hexokinases and glucokinase from *S. cerevisiae* were over-expressed in strains with a phosphorolytic pathway consisting of CDT-1(F213L) and the *S. degradans* CBP. While the over-expression of *HXK2* or *GLK1* did not change strain performance, over-expression of *HXK1* resulted in a 28% improvement in ethanol productivity (**Figure 5-13 and Table 5-14**). Furthermore, the ethanol productivity of this strain increased linearly with increasing initial cell densities, up to 2.69 g ethanol/L-h with an initial OD(600 nm) of 23.1 (**Figure 5-15, Table 5-16 and Figure 5-17**).

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 cellobiose transporter and an intracellular cellobiose phosphorylase. A similar strain was first reported by Sadie *et al.* (Sadie, et al., 2011). The Sadie *et al.* strain consumed ~10 g/L cellobiose in ~114 hours, 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 hours, producing ethanol with a yield of ~0.44 g/g. This rate matches that of previously described strains that share the same cellobiose transporter, but instead contained an intracellular cellobiose hydrolase (Ha, et al., 2011). Notably, cellobiose phosphorolysis leads to consistently higher yields than the hydrolytic pathway. We speculate that the improved yields are due to a lack of cellodextrin accumulatation 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 cellodextins 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 diphasic fermentation pattern: rapid early-stage cellobiose fermentation, followed by slow late-stage cellodextrin fermentation. In constrast, engineered strains with the

phosporolytic pathway showed a consistent cellobiose fermentation rate, without cellodextrin accumulation.

There is a profound energetic difference between the phosphorolytic and hydrolytic pathways. The phosphorolytic pathway begins with the phosphorolysis of cellobiose to glucose and Gluc-1P (ΔG^o = 3.6 kJ mol^{-1}). This Gluc-1P is converted to Gluc-6P by phosphoglucomutase (ΔG^o = -7.0 kJ mol^{-1} (Goldberg & Tewari, 1989)), while glucose is phosphorylated to Gluc-6P by hexokinase (-16.7 kJ mol^{-1} (Goldberg, 1975)) in a reaction that consumes 1 ATP. Thus, the overall free energy change associated with the phosphorolytic conversion of cellobiose to 2 Gluc-6P is approximately -20.1 kJ mol^{-1}, with 1 ATP consumed in the process. In contrast, the hydrolytic pathway begins with the hydrolysis of cellobiose to 2 glucose (ΔG^o = -12.5 kJ mol^{-1} (Tewari & Goldberg, 1989)), followed by the phosphorylation of this glucose by hexokinase (ΔG^o = -33.4 kJ mol^{-1} (Goldberg, 1975)). Thus, the overall free energy change associate with the hydrolytic conversion of cellobiose to 2 Gluc-6P is -45.9 kJ mol^{-1}, with 2 ATP consumed in the process.

The disparity in free energy change may explain why additional genetic modifications were necessary to obtain high fermentation rates in phosphorolytic strains. We propose that with a ΔG^o near unity, the phosphorolytic cleavage of cellobiose requires an additional thermodynamic "push" and "pull" to drive the reaction forward (**Figure 5-17**). This could explain why a strain evolved to rapidly consume cellobiose unexpectedly carried a transporter mutant with a high V_{max}. Our data suggests that the rate of cellobiose phosphorolysis is limited by low concentrations of substrate (cellobiose) and high concentrations of product (glucose, Gluc-1P). From this perspective, the high-V_{max} transporter that emerged from our selection and other high-V_{max} transporters likely increase the cellobiose consumption rate by raising intracellular cellobiose concentrations. This would "push" the phosphorolytic reaction into conditions where ΔG is negative. The benefit of over-expressing hexokinase in the phosphorolytic strain can also be understood in the same framework. Increased hexokinase activity would reduce intracellular glucose concentrations, "pulling" the phosphorolytic reaction into conditions where ΔG is negative.

The distinct response of the phosphorolytic pathway and the hydrolytic pathway to different transporter mutants may reflect that they are thermodynamically and kinetically limited, respectively. As discussed above, the energetics but not the kinetics of cellobiose phosphorolysis may limit the phosphorolytic pathway. This is in contrast to the hydrolytic pathway, which has more favorable energetics, and is more likely to be limited by enzyme kinetics. Our observation that increasing the V_{max} or intracellular concentration of the hydrolase raises fermentation rates supports this. It is therefore not surprising that unique transporter mutants would be optimal in the two scenarios; different transport parameters would be ideal in the two cases.

Since fermentations of lignocellulosic sugars are usually performed in oxygen-limited or anaerobic conditions, pathways with low ATP demands may be preferable for fuel and chemical production from lignocellulosic substrates. 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, Sedlak, Ho, & Mosier, 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.

In the present experiments, the theoretical increase in ATP yield when cellobiose is fermented through the phosphorolytic pathway did not have any observable consequences, besides a slight improvement in ethanol yield. In theory, only 1 ATP is consumed for every 2 Gluc-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. In contrast hydrolytic strains should yield 4 ATP per cellobiose fermented. Increased cellular ATP can lead to higher biomass yields per gram of carbon. This effect was documented by de Kok *et al.* (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. In contrast, we did not observe increased biomass yields during phosphorolytic cellobiose fermentation. We speculate that this difference may be due to culturing conditions, as de Kok *et al.* performed their fermentations in bioreactors with careful controls over growth under continuous culture conditions. Future experiments will be needed to demonstrate the benefit of increased ATP yield during fermentation of sugars derived from plant biomass hydrolyzates.

5.4 Materials and Methods

Cellobiose phosphorylase and phosphoglucomutase cloning

Cellobiose phosphorylase (CBP) genes from *Celvibrio gilvus* (CgCBP, Accession: AB010707), *Sacharophagus degradans* (SdCBP, Accession: YP_526792), and *Clostridium thermocellum* (CtCBP, Accession: YP_001036707) were codon-optimized (Figure SX) 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_pRS426) (Galazka, et al., 2010). The *C. gilvus* CBP was inserted using the primers, TTACTAGTATGGGATCATCTCACCACC and ATT CTGCAGTTA*ATGATGATGATGATGATG*TACTGTCACTTCGACTCTCACAGTAG, creating the plasmid PGK1_CgCBP_425. The *S. degradans* CBP was inserted using the primers, TTACTAGTATGAAATTCGGGCACTTTG and ATTCTGCAG*TTAATGATGATGATGATGATG*TCCAAGTGTTACCTCGACATTG, creating the plasmid PGK1_SdCBP_425. The *C. thermocellum* CBP was inserted using the primers, TTACTAGTATGAAGTTTGGCTTTTTCGATG and ATTCTGCAG*TTAATGATGATGATGATGATG*TCCAAGTGTTACCTCGACATTG, creating the plasmid PGK1_CtCBP_425. In all primers above and below restriction sites are underlined, while 6x-hisitidine tags are italicized.

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_pRS426 using the primers TTACTAGTATGTCATTTCAAATTGAAACGGTTC and ATTCTGCAGTTAAGTACGAACCGTTGGTTCTTC 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 using the primers ATGAGCTCTGAATAATACGACTCACTATAGGGCGAATTG and ATGAGCTCTGAATGGAAACAGCTATGACCATGATTACG. 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.

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, Nikawa, Kodaki, & Yamashita, 1992) using the yeast EZ-Transformation kit (BIO 101, Vista, Calif.). 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.

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 g/L of cellobiose in 250 mL flask at 30°C with initial OD_{600} of ~ 1.0 and under oxygen limited conditions. Cell growth was monitored by optical density (OD) at 600 nm using UV-visible Spectrophotometer (Biomate 5, Thermo, NY). Glucose, 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.

Directed evolution of phosphorolytic strains

A fermentation reaction was started as described above using the D452-2 strain of *S. cerevisiae* transformed with *cdt-1* and the *S. degradans* CBP. When cellobiose concentrations reached to almost zero, cells were collected and used to established new reactions at an OD (600 nm) of ~0.01. This process was repeated 7 times over the course of 30 days. At this point, cells were plated onto YSC plates containing 20 g/L of cellobiose to isolate clones. After the confirmation of improved phenotypes from isolated clones, plasmids were isolated from one of representative clone and sequenced.

Site directed mutagenesis and transporter kinetics

Site directed mutagenesis was performed using the Quickchange® protocol (Zheng, Baumann, & Reymond, 2004). The primers used to introduce each mutation are listed in the following table.

Mutation	*Sense primer (Mutated codon in lowercase)*	*Antisense primer (Mutated codon in lowercase)*
F213L	TCTACAACTGCGGTTGGttaGGAGGTTC GATTCC	GGAATCGAACCTCCtaaCCAACCGCAGTT GTAGA
G91A	TGCGCCAACGGTTACGATgcaTCACTCA TGACCGGAATCATC	GATGATTCCGGTCATGAGTGAtgcATCGT AACCGTTGGCGCA
F335A	GGTGCTCATGATCTCCATCgcaGGCCAG TTCTCCGGCAAC	GTTGCCGGAGAACTGGCCtgcGATGGAGA TCATGAGCACC
Q104A	ATCATCGCTATGGACAAGTTCgcaAACC AATTCCACACTGGTGAC	GTCACCAGTGTGGAATTGGTTtgcGAACT TGTCCATAGCGATGAT
F170A	TCCTCCAAGCTCGCTCAGgcaGTCGTTG GCCGCTTCGTT	AACGAAGCGGCCAACGACtgcCTGAGCGA GCTTGGAGGA
R174A	GCTCAGTTTGTCGTTGGCgcaTTCGTTC TTGGCCTCGGT	ACCGAGGCCAAGAACGAAtgcGCCAACGA CAAACTGAGC
E194A	GCCCCGGCCTACTCCATCgcaATCGCCCC TCCTCACTGG	CCAGTGAGGAGGGGCGATtgcGATGGAGT AGGCCGGGGC

Transporter kinetics were measured as described previously with slight modifications (Galazka, et al., 2010). Briefly, D452-2 transformed with mutant transporters was set-up at an OD (600 nm) of ~0.2 in 50 mLs of DOB –uracil and grown to an OD (600 nm) of ~1. Cells were then harvested by centrifugation, washed 3x with 10 mLs 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. To record linear rates of uptake over the course of 95 seconds, 50 µL of cells were added to 50 µL of [^{3}H]-cellobiose at the appropriate concentration with a final S.A. 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 minute at 17,000 g, 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®.

Measurement of GFP fluorescence during fermentations

During fermentations, when OD (600 nm) reached 10.0, they were harvested and washed twice by sterilized water. 200 µl of the cell suspension was transferred to a Corning black 96-well optical bottom plate (Corning, NY). Fluorescence intensities were measured with a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT) at an excitation wavelength of 485 nm, emission of 528 nm.

Measurement of enzymatic activity in cell extracts

Fermentation reactions were set-up 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 2x in 500 μL of ice-cold extraction buffer (50 mM HEPES-NaOH [pH 6.0], 2mM DTT, and Roche Complete EDTA-free Protease Inhibitor Cocktail), and resuspended in 200 μL of this buffer, this was moved to a screw-cap tube containing ~100 μL of 0.4 mm Zirconia/Silica beads. Cells were then lysed by bead-beating 3x for 30 s at 4 °C using a Biospec Products Mini-BEADBEATER WITH 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, Klei, & Sundstrom, 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, Bergmeyer, & Grassl, 1983). 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.

Purification of GH1-1, SdCBP, Hxk1, Hxk2 and Glk1

GH1-1 and SdCBP were purified directly from the D452-2 strains described above. 200 mL cultures in DOB –uracil –leucine were grown to an OD (600 nm) of ~7. Cells were pelleted and washed 1x with 40 mL ddH_2O. Cell pellets were resuspended in 25 mL of 50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 10 mM imidazole, 2 mM DTT, and Roche Complete EDTA-free Protease Inhibitor Cocktail, and lysed by passage through an Avestin EmulsiFlex C-3 homogenizer at 20,000 P.S.I. Cell debris was then pelleted, and proteins purified using Nickel-NTA agarose beads from Qiagen following the protocol for native batch purification provided by Qiagen. Protein eluted from the beads was buffer exchanged into a buffer consisting of phosphate buffered saline (PBS), 10% glycerol, and 2 mM DTT, snap-frozen in $(l)N_2$ and stored at -80 °C. Protein concentrations were determined by the absorbance at 280 nm using extinction coefficients of 108750 $M^{-1}\,cm^{-1}$ and 178540 $M^{-1}\,cm^{-1}$, for GH1-1 and SdCBP, respectively.

Hxk1 (Accession: NP_116711), Hxk2 (Accession: NP_011261), and Glk1(Accession: NP_009890) were expressed and purified in *E. coli*. First, genes were cloned into the PmlI and XhoI sites of the expression plasmid, pET302. Hxk1 was amplified with the primers,

CATTAACACGTGGTTCATTTAGGTCCAAAGAAACCAC and CATTAACTCGAGCAATGATACCAAGAGACTTACCTTCG. Hxk2 was amplified with the primers, CATTAACACGTGGTTCATTTAGGTCCAAAAAAACCAC and CATTAACTCGAGTTAAGCACCGATGATACCAACG. Glk1 was amplified with the primers, CATTAACACGTGTCATTCGACGACTTACACAAAGC and CATTAACTCGAGTCATGCTACAAGCGCACAC. These constructs were transformed into the BL21(DE3) strain of *E. coli*, and the proteins expressed and purified using Nickel-NTA agarose beads from Qiagen following the protocol for native batch purification provided by Qiagen. Protein eluted from the beads was buffer exchanged into a buffer consisting of 50 mM Tris-HCl [pH 8.0 at 30 °C], 13.3 mM $MgCl_2$, 2 mM DTT, and 10% glycerol, snap-frozen in (l)N_2 and stored at -80 °C. Protein concentrations were determined by the absorbance at 280 nm using extinction coefficients of 45840 M^{-1} cm^{-1}, 45840 M^{-1} cm^{-1} and 30370 M^{-1} cm^{-1}, for Hxk1, Hxk2 and Glk1, respectively.

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 hours, 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.

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®.

Effect of cellobiose on *S. cerevisiae* hexokinases

To measure the effect of high concentrations of cellobiose on the activity of purified Hxklp, Hxk2p and Glk1p, the activity of the purified proteins was measured in the presence or absence of 184 mM cellobiose, by coupling the production of glucose-6-phosphate to $NADP^+$ reduction through glucose-6-phosphate dehydrogenase in a manner identical to that used on cell extracts detailed above. Between 1 and 10 pmol of Hxk1, Hxk2 or Glk1 was used.

DNA sequences of codon-optimized cellobiose phosphorylases

>SdCBP

ATGAAATTCGGGCACTTTGATGATAAAGCAAGAGAGTACGTTATCACTGA
CCCAAAAACACCTTATCCTTGGATAAACTATCTTGGTAATGAGGACTTTT
TCTCATTGGTTTCTAATACAGGCGGTGGTTACTCCTTCTATAAGGACGCC
AAATTCAGGAGACTAACCAGATACAGATACAACAATGTTCCAGTCGATAA
TGGTGGCAAATACTTTTACATTAACGACTCTGGAGATGTGTGGTCACCAG

GATGGAAGCCTGTGAAGGCAGAACTAGATGCATACTCATGCGCTCATGGT
TTGAGTTATACTAGAATTACAGGTGAGAGAAATGGTATACAAGCCGAGGT
ACTTTCATTCATTCCATTAGGTACCTGGGCAGAAATACAAAAAGTGTCTT
TGAAAAACACATCTGGAGCCACCAAAAAGTTCAAACTGTTTTCATTTGCT
GAATGGTGTTTATGGAATGCAGAAGATGACATGACCAACTTTCAGAGAAA
CTTTTCCACCGGTGAAGTGGAGGTAGAAGATTCTGTCATCTACCATAAGA
CAGAGTTCAAAGAGCGTAGAAATCATTATGCTTTCTACAGTGTCAATGCA
CCAATACAGGGTTTCGATACTGATAGAGACAAATGGAAAGGCTTGTACAA
TGATTTTGACAAACCTGACGCAGTATTTGAAGGTGAACCTAGAAATTCAG
AAGCACACGGCTGGTCCCCTATCGCTTCCCATTATCTTGAAGTAGAATTG
GCCCCTGGGGAGTCTAAGGATCTTATCTTCGTATTAGGCTACATTGAAGT
CGCCCCTGAAAACAAATGGGAATCAAAAGGTGTTATCAACAAATCCCCAG
CCAAGGAACTAATCGCTCGTTTTGATTCTGTAGAGAAAGTGGATGCTGAA
TTGACTAAGTTAGCTGACTATTGGGCTAACTTGCTATCTACTTATTCCGT
TGAATCTGGGGATGAGAAACTGGACAGAATGGTTAACATTTGGAACCAAT
ACCAATGCATGGTAACATTCAATATGTCTAGATCTGCATCCTTTTTCGAG
TCAGGAATTGGCAGGGGCATGGGTTTTAGAGATTCTAATCAAGACTTGAT
AGGCTTTGTACACCAAGTACCAGAAAGAGCAAGAGAACGTATCATCGACA
TAGCTTCCACTCAATTTGAAGATGGATCTGCTTACCATCAATACCAGCCT
TTAACTAAAAGAGGCAATAACGCAATTGGGGGTAACTTTAACGATGATCC
ATTATGGTTGATTTTGAGTACCACTGATTACATCAAGGAAACTGGAGATT
TCTCCATTCTGGAGGAACAAGTTCCTTACGATAATGATGCCTCAAAGGCC
ACTTCACATTTTGAACACTTGAAAAGATCCTTTTACCATACTGTTAACAA
TCTTGGACCTCATGGCTTGCCACTGATCGGTAGAGCTGACTGGAATGATT
GCCTTAATCTGAATTGTTTTTCTGAAGATCCTAACGAGTCATTCCAGACT
ACAGGCAATAAGACAGGTAGAACAGCCGAGTCACTAATGATCGCCGGATT
GTTCGTGTTATACGGTAATGAATTTGTGAAATTGTGTCGTGAAATTGGTC
AAGACGGAGAAGCTGCCGAAGCACAAGCTCATATCGACCAAATGGTTGAA
GCCGTCAAAAAGCACGGATGGGACGGTGAATGGTTCCTTAGAGCTTACGA
TTACTACGGCAAAAAGGTTGGTTCTAAGGAAAATGAGGAAGGTAAGATTT
TCATTGAATCTCAAGGTTTTTGTGGTATGGCAGGGATTGGTTTAGAAGAT
GGTTTGGTTGAAAAGTCTATGGATTCTGTTAAGGAATGGTTAGATTGTGA
TTACGGTATAGTTCTACAACAGCCAGCTTTTACAAAGTACTATATCGAAT
ATGGTGAAATCTCAACTTATCCAGCCGGGTACAAAGAGAATGCTGGAATC
TTTTGTCATAACAACCCATGGATTATGATAACAGAAACACTATTAGGAAG
AGGCGACAAGGCTTTCGAGTACTATAGGAAAATCGCACCTGCATACCTGG
AAGAGATTTCTGACTTGCATAAAGTGGAACCATACGCTTATTGTCAAATG
ATAGCTGGAAAAGATGCTTACTTACCAGGAGAAGGCAAAAACAGTTGGTT
AACAGGGACTGCATCATGGAATTTCGCTGCTATTACACAGTACATTCTAG
GTGTCAAACCAGATTACTCTGGGTTGGCTATCAACCCATGTATTCCATCT
AGTTGGGATGGATTCAAAGTCACCAGAAAGTACCGTGGGGCAACTTACAA
CATAATCGTGACAAATCCAACTCACGTTTCTAAGGGGGTTAAGTCATTAA
CACTGAATGGCAATGCCATCGACGGCTACATCGTCCCACCACAACAAGCA
GGTACCGTTTGCAATGTCGAGGTAACACTTGGATAA

>CgCBP

ATGGGATCATCTCACCACCATCATCATCATTCCTCAGGTCTTGTTCCTAG
AGGATCACATATGAGGTACGGACATTTCGATGATGCAGCAAGAGAATACG
TTATCACTACTCCACACACTCCTTATCCTTGGATTAACTATCTGGGCTCT
GAACAGTTTTTCTCTCTGTTATCTCATCAAGCTGGGGGATACTCTTTCTA
TAGGGATGCAAAGATGAGAAGATTGACAAGATACAGATACAACAATATCC
CTGCCGATGCAGGTGGAAGATACCTATACGTTAATGACGGTGGCGACGTT
TGGACCCCATCATGGCTACCAGTTAAAGCTGACCTTGACCATTTTGAAGC
TCGTCATGGACTGGGCTATAGTAGAATTACCGGCGAAAGGAACGGGCTAA
AAGTTGAAACATTGTTTTTCGTACCATTAGGCGAGAATGCAGAAGTACAG
AAGGTAACAGTCACAAATACTTCTGATGCTCCAAAAACTGCCACACTTTT
CTCTTTTGTCGAGTTTTGCCTATGGAATGCTCAAGATGATCAAACCAATT
ACCAGAGAAACTTGTCTATCGGTGAGGTCGAAGTGGAACAAGATGGTCCA
CATGGCTCTGCAATCTACCATAAGACAGAATACAGAGAACGTAGAGATCA
CTACGCCGTGTTTGGTGTCAATACAAGAGCTGATGGATTTGACACAGACA
GAGATACCTTTGTAGGTGCCTACAATTCACTTGGAGAAGCATCCGTTCCA
AGAGCCGGTAAGTCCGCTGATTCAGTCGCCTCTGGATGGTATCCTATAGG
TTCACATAGTGTAGCCGTTACTTTGCAACCTGGCGAGTCCAGAGATTTGG
TCTATGTGTTGGGCTACTTAGAAAACCCTGATGAAGAGAAATGGGCTGAC
GATGCACACCAAGTGGTTAACAAAGCACCAGCACACGCTCTGTTAGGTAG
GTTCGCTACATCTGAACAAGTTGACGCTGCACTAGAAGCATTGAACTCTT
ATTGGACAAACTTATTGAGTACTTACTCCGTTTCATCCACTGACGAAAAG
CTGGACAGAATGGTGAATATCTGGAATCAATACCAATGTATGGTTACTTT
CAATATGTCAAGATCAGCATCCTTTTTCGAGACAGGTATTGGCCGTGGAA
TGGGCTTTAGAGACTCTAATCAAGACCTTTTAGGTTTCGTGCATCTTATA
CCTGAACGTGCTAGAGAAAGGATCATTGATATTGCATCAACACAGTTTGC
AGATGGTTCAGCATATCATCAGTACCAACCTTTAACTAAAAGAGGTAACA
ACGACATAGGTTCCGGGTTTAACGATGATCCATTGTGGCTAATCGCTGGC
GTAGCTGCCTATATCAAAGAGTCTGGTGATTGGGGTATTCTAGATGAGCC
AGTTCCATTCGATAATGAACCAGGGTCTGAAGTGCCACTATTCGAACACT
TAACTAGATCTTTTCAATTCACCGTTCAGAATAGAGGACCACATGGGTTA
CCATTGATTGGCAGAGCCGACTGGAATGATTGTTTGAATCTTAACTGTTT
CTCAACTACACCAGGAGAGTCTTTTCAAACTACAGAAAACCAGGCTGGAG
GTGTCGCAGAGTCTGTTTTCATAGCTGCTCAATTTGTGTTGTATGGCGCA
GAATATGCTACTTTAGCAGAAAGGAGAGGTTTGGCTGATGTTGCTACTGA
GGCTAGAAAGTACGTGGACGAGGTTAGAGCTGCTGTGTTGGAACACGGTT
GGGATGGTCAATGGTTTTTGAGAGCCTACGACTACTACGGAAATCCAGTT
GGTACCGATGCCAAACCAGAGGGAAAGATCTGGATAGAACCACAAGGGTT
TGCCGTAATGGCAGGCATTGGTGTAGGTGAGGGCCCTGATGATGCCGATG
CCCCTGCCGTCAAGGCTTTAGATAGTGTTAACGAGATGTTAGGCACACCA
CATGGTCTTGTCCTGCAATACCCTGCATACACTACTTACCAAATAGAACT
GGGGGAAGTATCTACTTACCCTCCTGGATACAAGGAAAATGGTGGTATCT
TTTGCCACAATAACCCATGGGTTATCATAGCTGAAACTGTTGTCGGTAGA
GGGGCTCAAGCATTTGATTACTATAAGAGAATTACTCCTGCCTACAGAGA
GGACATCTCAGATACACACAAATTGGAACCATATGTTTATGCTCAAATGA
TTGCTGGAAAGGAAGCTGTCCGTGCAGGGGAAGCCAAAAACTCATGGTTA
ACCGGTACCGCTGCATGGAACTTTGTCGCTGTATCTCAATACCTACTTGG

TGTCAGGCCAGACTACGATGGCCTAGTTGTCGATCCTCAAATTGGGCCAG
ACGTGCCAAGTTACACAGTTACCCGTGTAGCAAGAGGTGCTACTTATGAA
ATCACAGTTACAAATAGTGGTGCTCCTGGAGCTAGAGCTAGTTTGACAGT
AGACGGCGCTCCTGTTGATGGGAGAACAGTTCCTTACGCCCCAGCAGGAT
CTACTGTGAGAGTCGAAGTGACAGTA TAA

>CtCBP
ATGAAGTTTGGCTTTTTCGATGATGCCAATAAGGAATATGTTATCACAGT
GCCAAGGACACCTTATCCTTGGATTAACTATCTAGGAACTGAAAACTTTT
TCTCTCTTATCAGTAATACCGCAGGTGGCTACTGCTTCTATAGGGACGCA
AGATTACGTAGAATTACTCGTTACAGATACAACAATGTTCCAATTGACAT
GGGAGGTAGGTACTTCTATATCTACGATAACGGTGATTTCTGGAGTCCAG
GATGGTCACCAGTGAAGAGAGAATTGGAATCATATGAATGTCGTCATGGC
TTGGGATACACAAAGATAGCCGGTAAGAGAAATGGCATTAAGGCAGAAGT
TACCTTTTTCGTTCCACTGAATTACAATGGCGAAGTGCAGAAACTGATCT
TGAAAAACGAAGGTCAAGACAAAAAGAAGATTACACTGTTTTCATTCATT
GAGTTTTGTTTGTGGAATGCTTACGATGACATGACCAACTTTCAAAGGAA
TTTCTCTACAGGAGAGGTCGAGATTGAAGGGTCAGTTATCTACCACAAAA
CAGAGTATAGAGAACGTAGAAATCATTACGCCTTTTACTCAGTCAACGCT
AAGATTTCTGGGTTTGATTCCGACAGAGATTCATTCATCGGTCTATACAA
TGGATTTGATGCTCCACAAGCCGTCGTTAATGGTAAGTCCAACAATTCAG
TCGCAGATGGTTGGGCACCTATAGCTTCCCACTCTATTGAGATCGAACTT
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CAAGGACGAAGAGAAGTGGGAATCCAAAGGCGTGATAAACAAAAAGAAAG
CCTATGAGATGATAGAACAGTTTAACACAGTAGAAAAAGTAGACAAAGCC
TTCGAGGAATTGAAATCATATTGGAATGCACTGTTATCAAAGTATTTCCT
AGAATCCCATGATGAAAAGTTAAACAGAATGGTGAATATCTGGAATCAAT
ATCAATGCATGGTTACCTTTAACATGAGTAGATCTGCATCTTACTTTGAA
TCTGGTATAGGTAGAGGGATGGGCTTTAGAGATTCTAATCAAGACCTGCT
TGGTTTCGTTCATCAAATTCCTGAAAGAGCTAGAGAAAGGTTGCTTGATC
TAGCTGCCACTCAGTTGGAAGATGGTGGTGCATACCATCAATACCAGCCA
TTAACCAAAAAGGGGAACAATGAGATTGGAAGTAACTTTAACGACGACCC
TTTATGGTTAATCCTAGCAACTGCTGCTTACATCAAAGAGACAGGAGATT
ACTCAATACTTAAAGAGCAAGTTCCATTCAACAATGATCCATCTAAAGCC
GATACTATGTTTGAACATCTGACTAGATCTTTCTACCACGTAGTAAACAA
TCTTGGACCACACGGTTTACCACTAATCGGTAGAGCTGACTGGAACGATT
GTTTGAATTTGAATTGCTTCTCTACTGTACCTGATGAAAGTTTCCAAACT
ACAACATCAAAAGATGGGAAAGTTGCAGAATCTGTAATGATAGCTGGCAT
GTTCGTGTTTATCGGCAAAGATTATGTCAAGTTGTGCGAATACATGGGTC
TTGAAGAGGAAGCTAGAAAAGCACAACAACATATTGATGCTATGAAAGAG
GCTATCTTAAAGTACGGATACGACGGTGAATGGTTTTTGAGAGCTTATGA
CGACTTCGGGAGAAAAGTCGGTTCTAAAGAGAACGAGGAAGGAAAGATCT
TTATCGAATCCCAAGGTTTTTGTGTTATGGCCGAAATAGGCTTAGAAGAT
GGAAAAGCACTAAAGGCTCTAGATTCTGTCAAAAAGTATTTGGATACACC
ATACGGCTTAGTTTTACAGAATCCTGCATTCACAAGATACTACATTGAAT
ACGGCGAAATCAGTACTTACCCACCAGGTTACAAGGAAAATGCTGGCATC

TTTTGTCATAACAATGCTTGGATTATCTGCGCTGAGACAGTAGTTGGCAG
AGGTGATATGGCTTTTGATTACTACAGAAAGATCGCACCTGCTTACATTG
AGGACGTTTCTGATATTCACAAGTTGGAACCATATGTGTACGCACAAATG
GTAGCAGGTAAGGACGCCAAAAGACATGGGGAAGCCAAAAACTCTTGGCT
GACTGGTACAGCCGCTTGGAATTTCGTAGCCATTTCCCAATGGATATTGG
GTGTTAAACCAGATTACGATGGATTGAAAATCGACCCTTGTATTCCAAAA
GCATGGGATGGTTACAAAGTCACTAGATACTTTAGAGGTTCTACCTACGA
AATCACAGTGAAAAACCCAAATCATGTCTCCAAAGGTGTTGCAAAGATAA
CAGTTGATGGGAACGAGATCTCAGGAAACATCTTGCCAGTTTTCAATGAT
GGCAAGACTCATAAAGTAGAAGTGATTATGGGATAA

6. Comments on sugars sensing by *S. cerevisiae* during growth on cellobiose

Partially taken from: Galazka, J. M., & Cate, J. H. (2011). A new diet for yeast to improve biofuel production. *Bioeng Bugs*, *2*(4).

6.1 Abstract

Recently we announced the discovery of two cellodextrin transporter families from the cellulolytic fungus, *Neurospora crassa*. Furthermore, we demonstrated the utility of these transporters in the production of lignocellulosic biofuels. This discovery was made possible by a decision to systematically study cell wall degradation by *N. crassa*. The identified transport pathway has opened up a new way of thinking about microbial fermentation of hexoses as well as pentoses derived from plant cell walls. Integrating this pathway with the endogenous metabolism and signaling networks of *Saccharomyces cerevisiae* is now a major goal of our group.

6.2 Discussion

Developing methods to convert the sugar-rich cell walls of plants to fuel is a major societal goal (Himmel et al., 2007), and has become the focus of intense research (Kalluri & Keller, 2010). Present methods for conversion are limited by the evolved recalcitrance of plant cell walls to depolymerization into constituent sugars, and deficiencies in the traits of microbes used to ferment these sugars to fuels such as ethanol (United States. Dept. of Energy. Office of the Biomass Program. & United States. Dept. of Energy. Genomics:GTL., 2006). It is our philosophy that fundamental breakthroughs addressing both of these limitations will be made through the study of model organisms. Hence, we study the mechanisms by which the filamentous fungus, *Neurospora crassa*, degrades and metabolizes plant cell walls. *N. crassa* has been studied since the 1920's (Perkins, 1992), and features a wealth of resources including a high quality genome (Galagan, et al., 2003), facile methods for genetic manipulation (Dunlap et al., 2007), an almost complete set of gene knockouts (Colot, et al., 2006), and an international community of researchers. In the wild, *N. crassa* is commonly found growing upon recently burned plant matter (Perkins, Turner, & Barry, 1976), and in 1977 was reported to secrete cellulases in response to specific inducers (Eberhart, et al., 1977). However, until 2009 *N. crassa* was largely ignored as a model biomass degrader. Research focused instead on the related fungus, *Trichoderma reesei* (*Hypocrea jecorina*). *T. reesei* is now favored by industry for its ability to secrete large quantities of cellulases used to deconstruct plant biomass (Seidl, et al., 2008).

Biomass degradation by *N. crassa* was revisited in 2009, with the report of a systematic study of plant cell wall degradation by this fungus (Tian, et al., 2009). Transcriptomic, proteomic, and phenotypic data showed: (i) that the core cellulases of *N. crassa* are similar to those of *T. reesei*; (ii) that *N. crassa* transcriptionally upregulates ~2% of genes in response to pure cellulose or plant cell walls; (iii) that there is a large amount of functional redundancy to the cellulase system of *N. crassa*, as only a cellobiohydrolase(I)

deletion strain had a significant deficiency in its capacity to degrade pure cellulose. Interestingly, the set of cellulose-induced genes identified in this study included 10 major facilitator superfamily transporters. The strong induction of these transporters during biomass degradation led to a hypothesis that they have a key role in this process. In addition to the transporters, we noticed that *N. crassa* induced the expression of a putative intracellular β-glucosidase, an enzyme that cleaves the β-1,4-glycosidic bond in short cellodextrin chains. Cellodextrins are β-1,4-linked oligosaccharides of glucose, and are the product of cellulose depolymerization by fungal cellulases (Y. H. Zhang & Lynd, 2004). We therefore reasoned that a likely substrate for some of these transporters were cellodextrins.

In 2010, we showed that two transporters, CDT-1 and CDT-2, indeed mediate the uptake of cellodextrins by *N. crassa*, and that following uptake cellodextrins are hydrolyzed intracellularly to glucose by a β-glucosidase (Galazka, et al., 2010). A number of transcriptomic studies have shown that orthologs of these transporters are transcriptionally upregulated in response to plant cell wall material or cellobiose in diverse fungi, suggesting they are fundamental to the strategies used by fungi to interact with plants (Martin, et al., 2010; Noguchi, et al., 2009; Vanden Wymelenberg et al., 2010). The identification of these genes confirmed reports of cellobiose permeases (Kubicek, Messner, Gruber, Mandels, & Kubicek-Pranz, 1993) and intracellular β-glucosidases (M. Saloheimo, et al., 2002) in *T. reesei*.

The importance of these transporters to biomass degradation by *N. crassa* and other fungi inspired us to engineer the transport pathway into the yeast, *Saccharomyces cerevisiae*. *S. cerevisiae* is the current organism of choice for fermenting glucose and sucrose to ethanol, but lacks the capacity to ferment cellodextrins (Lagunas, 1993). Strains engineered to express *cdt-1* or *cdt-2* along with an intracellular β-glucosidase (*gh1-1*) grew with cellodextrins as a sole carbon source, and fermented cellobiose to ethanol efficiently. Multiple reports preceded ours in describing cellodextrin consumption or fermentation by engineered strains of *S. cerevisiae*. As early as 1986, Kohchi & Toh-e reported the consumption of cellobiose by *S. cerevisiae* expressing a secreted β-glucosidase from *Candida pelliculosa* (Kohchi & Toh-e, 1986). They did not measure its fermentative capacity, however. Then, in 1988 Machida et al. reported cellobiose fermentation by *S. cerevisiae* expressing secreted β-glucosidases from *Saccharomycopsis fibuligera* (Machida, et al., 1988). Subsequent studies have repeated this feat both in lab (Benoliel, et al., 2010; Kotaka, et al., 2008b; Murai, et al., 1998; Rajoka, et al., 1998; Tokuhiro, et al., 2008; Uryu, et al., 2006; Van Rensburg, et al., 1998; van Rooyen, et al., 2005) and industrial strains of *S. cerevisiae* (Saitoh, et al., 2008; Shen, et al., 2008), and in other yeast species (Yanase et al., 2010). All of these strains rely on extracellular hydrolysis of cellobiose to glucose by secreted β-glucosidases followed by uptake *via* the endogenous *S. cerevisiae* hexose transporters. In contrast, the first step in the pathway we described is the import of cellodextrins into the cytosol of *S. cerevisiae*, only then does hydrolysis to glucose occur. Other yeasts in nature have been shown to ferment cellobiose, but the molecular basis for this capability was not determined (S. N. Freer, 1991; Shelby N. Freer & Detroy, 1982; S. N. Freer & Greene, 1990; Preez, Bosch, & Prior, 1986).

There are two main distinctions between cellodextrin fermentation mediated by transport and one mediated by extracellular hydrolysis to glucose: (i) cellodextrin transporters have a relatively high affinity for cellodextrins; (ii) cellodextrins are hydrolyzed intracellularly. Both may be important during the fermentation of plant cell wall derived sugars. The high-affinity of CDT-1 and CDT-2 for cellodextrins could be particularly important during the simultaneous saccharification and fermentation (SSF) of plant cell walls to fuel. SSF, in which fermenting microbes are included in the depolymerization reaction, increases the efficiency of conversion by relieving product inhibition upon cellulases (Doran-Peterson, et al., 2009). With a K_M of ~5 μM, cellodextrin transporters may be capable of reducing sugar concentrations below levels obtainable by the inclusion of extracellular β-glucosidases, which generally have a K_M of 100 – 1000 μM (Chauve, et al., 2010). This idea has not been rigorously tested and further studies applying and validating models of SSF using cellodextrin-transporting strains are necessary (Levine, Fox, Blanch, & Clark, 2010; van Zyl, van Rensburg, van Zyl, Harms, & Lynd, 2010).

Relocating hydrolysis of cellodextrins from outside to inside the cell may seem like a trivial change, but it has a profound advantage. Specifically, transport of cellodextrins followed by intracellular hydrolysis to glucose facilitates the cofermentation of cellulose-derived glucose and hemicellulose-derived xylose (Ha, et al., 2011; Li, et al., 2010). The inability of *S. cerevisiae* to coferment glucose and xylose is a barrier to efficient lignocellulosic biofuel production. Both glucose and xylose enter *S. cerevisiae* through the endogenous *S. cerevisiae* hexose transporters, but glucose is vastly preferred to xylose (Kötter & Ciriacy, 1993; W. J. Lee, et al., 2002). Thus, xylose will not be taken up until all glucose is consumed. This sequential fermentation of glucose and xylose leads to lower ethanol yields and productivities (Kim, Block, & Mills, 2010b). Cellodextrin transport represents an alternate route for glucose to enter yeast, leaving the endogenous hexose transporters unoccupied and capable of transporting xylose. Strains containing the cellodextrin transport pathway from *N. crassa* and an efficient xylose fermentation pathway coferment cellobiose and xylose at high rates and with high yields (Ha, et al., 2011; Li, et al., 2010).

Intracellular hydrolysis of cellodextrins does have associated problems, however. Yeast set metabolic and growth rates to match the perceived abundance of nutrients (Slattery, Liko, & Heideman, 2008; Zaman, Lippman, Schneper, Slonim, & Broach, 2009). Normally, perceived and actual nutrient availabilities match, and metabolism is optimal. This may no longer be the case in strains engineered to hydrolyze cellodextrins intracellularly. *S. cerevisiae* detects the presence of extracellular glucose *via* three sensors: the 7-transmembrane receptor, Gpr1; and two non-transporting transceptors, Rgt2 and Snf3 (**Figure 6-1**). The associated signaling pathways induce a robust transcriptional response to glucose, placing *S. cerevisiae* in an optimal state for high-capacity fermentation (Goncalves & Planta, 1998). In strains engineered to hydrolyze cellodextrins intracellularly, glucose is never present at high concentrations outside of the cell (though some glucose may leak out through the endogenous *S. cerevisiae* hexose transporters). Therefore, the presence of glucose in the form of cellodextrins will not be signaled through Snf3, Rgt2 & Gpr1, placing the cell in a sub-optimal state. Additional signals emanate from intracellular glucose, and presumably these pathways will still respond following

hydrolysis of transported cellodextrins as is seen following the transport and hydrolysis of maltose (Rolland et al., 2000). Presently, we do not know if strains engineered to transport and hydrolyze cellodextrins respond appropriately to the presence of glucose in the form of cellodextrins. This will be an important area of future experimentation, the results of which may necessitate the integration of cellodextrin metabolism into the signaling network of *S. cerevisiae*.

The identification of a cellodextrin transport pathway in *N. crassa* has offered insights into the strategies used by filamentous fungi to degrade plant cell walls, and the successful recapitulation of this pathway in *S. cerevisiae* has provided new routes for the microbial synthesis of lignocellulosic fuels. Many questions must be answered before this pathway is fully integrated with the endogenous metabolism of *S. cerevisiae*, and additional development will be necessary to produce a strain useful in industrial processes.

7. Discovery and Application of Cellodextrin Transporters

Partially taken from: Galazka, J. M., & Cate, J. H. (2011). Improving the bioconversion of plant biomass to biofuels: A multidisciplinary approach. *Energy & Environmental Science.*, *4*(9), 3329.

7.1 Abstract

In 2010 our group reported the discovery of two cellodextrin transporter families, and soon after demonstrated the utility of these transporters in the production of lignocellulosic biofuel. These discoveries required diverse insights from multiple research groups, highlighting the need for multidisciplinary teams to tackle the most pressing research problems in bioenergy.

7.2 Discussion

Developing alternatives to petroleum-derived liquid transportation fuels has become a prime societal goal due to their rising direct and indirect costs (Shinnar & Citro, 2006). Two promising biological replacements are lipids derived from microalgae (Scott et al., 2010), and the sugars within the cell walls of plants(Carroll & Somerville, 2009). Both of these can be converted to liquid fuels, and critically this conversion does not necessarily put upward pressure on global food prices (Somerville, et al., 2010; Tilman et al., 2009). Our own work targets the sugars within plant cell walls, and the remainder of this perspective will recount our recent progress towards enabling their efficient conversion to a suitable transportation fuel.

While the sugars within plant cell walls represent an abundant energy source (Somerville, et al., 2010), accessing them is difficult; they are bound within a complex network consisting mainly of cellulose (a polymer of glucose), hemicellulose (predominantly a polymer of xylose), and lignin (a complex phenolic polymer) that has evolved significant resistance to degradation. Despite this, there are a number of ways to convert this material to liquid fuels (Carroll & Somerville, 2009). For example, it is possible to thermally convert plant cell walls to a gas stream composed of syn-gas (CO and H_2) as well as other gases such as CO_2, CH_4, and N_2. The technology for chemically converting syn-gas into a liquid fuel is well established (Huber, et al., 2006). Our focus, however, is on a bioconversion process relying first upon the enzymatic depolymerization of plant cell walls into soluble sugars. The free sugars are then converted to liquid fuels either chemically (Huber, et al., 2005), or *via* microbial fermentation by yeasts or bacteria (Stephanopoulos, 2007).

Currently, none of these methods results in a market-competitive fuel (Aden & Foust, 2009; Sims, et al., 2010), and technological barriers must be overcome (U.S. Dept. of Energy, 2006). A number of investments have been made to understand these barriers and

to identify possible solutions (Kalluri & Keller, 2010). One example is the establishment of the Energy Biosciences Institute (EBI), a research collaborative between the University of California Berkeley, Lawrence Berkeley National Laboratory and the University of Illinois Urbana-Champaign, funded by British Petroleum (BP) (Kintisch, 2007). The groups within this institute, including our own, span a range of disciplines including agronomy, chemical engineering, plant and fungal biology, and socioeconomics. The breadth of the EBI allows for serendipitous encounters and unique collaborations, which will be evident in this perspective as we share our experiences of the last ~2 years.

In 2006, our group began to develop the anaerobic bacterium, *Clostridium papyrosolvens C7*, as a model system to understand bacterial degradation of plant biomass, in collaboration with the lab of Michael Marletta. *C. papyrosolvens C7* depolymerizes biomass with large multi-enzyme complexes called cellulosomes (Fontes & Gilbert, 2010; Pohlschroder, Canale-Parola, & Leschine, 1995). Some have suggested that tethering enzymes within cellulosomes allows a level of synergism impossible to obtain otherwise(Warren, 1996), though this remains an open question (Ng & Zeikus, 1981). As part of our efforts to understand cellulosome function, William Beeson began developing an assay to rapidly measure the enzymatic activity of cellulosomes purified from *C. papyrosolvens C7* cultures. The assay coupled the cellulosome-catalyzed release of soluble glucan chains to the reduction of a dye *via* cellobiose dehydrogenase (CDH, EC 1.1.99.18). This enzyme was previously purified from the secretome of the fungus, *Myceliophthora thermophila* (*Sporotrichum thermophile)*, another plant biomass degrading organism (Canevascini, 1988). Purifying enough CDH for the number of assays we intended to run required growing large quantities of *M. thermophila*. A 10 liter flask of *M. thermophila* growing on plant biomass is a dramatic sight, and it caught the eye of the current director of the EBI, Chris Somerville. He knew that Louise Glass, a UC Berkeley faculty member and a member of the EBI, was studying a related fungus, *Neurospora crassa*. Realizing that our interests overlapped, and that our three labs had complementary skill sets and knowledge bases, he helped the groups launch a collaborative analysis of plant biomass degradation by *N. crassa*. Notably, the collaboration between the labs was well underway before the principle investigators (Cate and Glass) had personally met.

A National Institutes of Health model organism, *N. crassa* was found to be cellulolytic in 1949 (Marsh, et al., 1949). However, this aspect of its biology was not deeply investigated as interest focused on *Hypocrea jecorina* (*Trichoderma reesei*), a fungus favored by industry for its ability to secrete large amounts of cellulases (Seidl, et al., 2008). To establish *N. crassa* as a model plant biomass-degrader, the three EBI labs performed a systems level study of the genes and proteins important to plant cell wall depolymerization and metabolism using a combination of genetics, transcriptomics, proteomics and biochemistry (Tian, et al., 2009). This study established that ~2% of the ~10,000 genes in *N. crassa* are transcriptionally upregulated in response to pure cellulose or plant biomass. A number of these are orthologous to those first studied *H. jecorina*. For example, to depolymerize cellulose, *N. crassa* transcriptionally upregulates a core set of enzymes consisting of glycosyl hydrolase family 6 (*gh6-2*, EC 3.2.1.91) & 7 (*cbh-1*, EC 3.2.1.-) cellobiohydrolases that hydrolyze cellulose chains processively from each end, and various endoglucanases that hydrolyze glycosidic linkages in the middle of chains. The product of

these cellulases is largely cellobiose, a β-1,4-linked dimer of glucose, although cellotriose and cellotetraose are also produced. The produced cellodextrins are hydrolyzed to glucose by β-glucosidases (EC 3.2.1.21).

Among the cellulose-responsive genes of *N. crassa*, 10 predicted major facilitator superfamily (MFS) transporters were upregulated on plant biomass. Their apparent utility during cellulose degradation led us to wonder whether they were responsible for transporting the cellodextrins created by the cellulases of *N. crassa* across the plasma membrane for intracellular metabolism. We suspected at least some might be involved in cellodextrin transport, because *N. crassa* also upregulated a predicted intracellular β-glucosidase when growing on cellulose, an enzyme that would only be needed for hydrolysis of intracellular glucan chains. Another hint came from the work of Kubicek *et al.*, who reported a cellobiose transport activity in *H. jecorina* in 1993 without identifying the responsible gene (Kubicek, et al., 1993). The availability of an almost complete gene-deletion set in *N. crassa* (Dunlap, et al., 2007) allowed us to rapidly screen the phenotype of strains in which the upregulated transporters were ablated. But these analyses were complicated by redundancy within the *N. crassa* transport system, and we were unable to draw strong conclusions about the function of individual transporters.

One of us (JMG) noted that *Saccharomyces cerevisiae* lacks the complexity of *N. crassa* as it lacks a permease for cellobiose or other cellodextrins (Lagunas, 1993), and does not produce a β-glucosidase. Thus, we reasoned that a functional cellodextrin transporter would allow yeast to accumulate cellodextrins intracellularly, where they could be hydrolyzed to glucose by an intracellular β-glucosidase (also from *N. crassa*), allowing *S. cerevisiae* to grow on an otherwise unusable carbon source. With cellobiose-mediated growth of *S. cerevisiae* as our assay, we showed that two *N. crassa* genes, *cdt-1* and *cdt-2*, encode cellodextrin transporters (Galazka, et al., 2010). In addition, we showed that the engineered yeast strains could efficiently transport cellobiose and, following its intracellular hydrolysis, ferment the resulting glucose to ethanol, a desirable trait as it alleviates the need to add extracellular β-glucosidase to cleave cellobiose to glucose.

A number of studies have now documented the importance of *cdt-1* and *cdt-2* orthologs to the lifestyle of fungi that live on plants. In *Aspergillus oryzae*, overexpression of XlnR, the key transcriptional activator of cellulases and hemi-cellulases in this organism, results in a 7-fold transcriptional induction of a *cdt-2* ortholog (Noguchi, et al., 2009). Among the 74 other genes upregulated by this manipulation, 32 were glycosyl hydrolases including cellulases and hemi-cellulases. Likewise, in expression microarrays performed on *Phanerochaete chrysosporium* and *Postia placenta* grown on ball-milled aspen, orthologs of *cdt-2* were upregulated 3-fold and 6-fold as compared to glucose-grown cultures, respectively (Vanden Wymelenberg, et al., 2010). Perhaps most intriguingly, when the ectomycorrhizal plant symbionts, *Tuber melanosporum* and *Laccaria bicolor*, are in contact with plant roots, orthologs of *cdt-1* are upregulated 35-fold and either 5- or 8-fold, respectively, as compared to free-living controls (Martin, et al., 2010).

While we don't understand the role these orthologs play in the lifestyle of these species, their induction in such diverse species–orthologs of *cdt-2* are used by Ascomycetes

and Basidiomycetes even after ~500 million years of divergence (Stajich, et al., 2009)– suggests they are fundamental to interactions between plants and fungi. In the wild, celluloytic fungi like *N. crassa*, *A. oryzae*, *P. chrysosporium*, and *P. placenta* may use high affinity cellodextrin transporters to efficiently scavenge sugars produced by their secreted cellulases away from competing organisms. Also, these transporters may enhance the activity of secreted cellulases by reducing product inhibition of cellulases by cellodextrins. Indeed, in a given species, the kinetics of cellodextrin transport would have co-evolved with the kinetics of cellulose hydrolysis, reiterating the need to consider the enzymes used by a particular species holistically (Bhat & Maheshwari, 1987; Levine, et al., 2010).

As first suggested by Kubicek *et al.* (Kubicek, et al., 1993), cellodextrin transporters may be critical to cellulose sensing. In one possible model, constitutively secreted cellulases produce low levels of cellodextrins, which enter the cell *via* a cellodextrin transporter, causing the induction of genes necessary for optimal cellulose degradation (Messner & Kubicek, 1991). Indeed, a recent study of *P. chrysosporium* showed that cellotriose and cellotetraose were strong inducers of cellulase genes (Samejima, Suzuki, & Igarashi, 2010). It is reasonable to expect that orthologs of CDT-1 and/or CDT-2 are involved in this process, but this is speculation.

The role of these transporters in *T. melanosporum* and *L. bicolor* during symbiosis with plants is mysterious. Ectomycorrhizal fungi are not thought to hydrolyze and consume large amounts of cellulose, instead primarily relying upon plant-provided sucrose as a carbon and energy source (Nehls, Grunze, Willmann, Reich, & Kuster, 2007). One explanation may be that these transporters have evolved to transport sucrose in these fungi, but this would conflict with a standard model in which glucose and fructose are taken up by ectomycorrhizal fungi following sucrose hydrolysis by plant invertase (EC 3.2.1.26) (Plett & Martin, 2011). Indeed, *L. bicolor* does not encode an invertase gene capable of hydrolyzing transported sucrose (Martin et al., 2008), and while *T. melanosporum* contains invertase, it is predicted to be secreted (Ceccaroli et al., 2011; Martin, et al., 2010). Notably, during mutualistic growth, *T. melanosporum* and *L. bicolor* produce glycosyl hydrolase family 5 cellulases (Martin, et al., 2010). Although the role of these enzymes in this context is unclear, they could be used to soften the plant cell wall while structures necessary for nutrient exchange between fungus and plant develop (Plett & Martin, 2011). When these cellulases are active, high affinity cellodextrin transport may be important to avoid cellodextrin accumulation, as cellodextrins are capable of inducing the plant innate immune response (Aziz et al., 2007).

Shortly after the discovery of these transporters, BP scientist Xiaomin Yang, realized that they would be useful to a longstanding goal of the yeast biotechnology field: the integration of hexose and pentose fermentation pathways into one strain. In contrast to other yeast species such as *Scheffersomyces stipitis* (*Pichia stipitis*) (Agbogbo & Coward-Kelly, 2008), *S. cerevisiae* does not naturally ferment pentose sugars to ethanol (Kim, et al., 2010b). This is problematic, as up to 28% of lignocellulosic biomass is composed of pentoses, mostly as xylose (J. Lee, 1997; Olsson & HahnHagerdal, 1996), and it must be fermented for lignocellulosic biofuels to be economically viable (Hinman, Wright, Hoagland, & Wyman, 1989). This limitation has been overcome by engineering strains to

express either xylose reductase (EC 1.1.1.307) and xylitol dehydrogenase (EC 1.1.1.9) together, or xylose isomerase (EC 5.3.1.5) alone, allowing high xylose fermentation rates (Karhumaa, et al., 2007; Kotter, et al., 1990; Kuyper, et al., 2003; Pitkanen, Rintala, Aristidou, Ruohonen, & Penttila, 2005). Despite this, xylose could not be fermented with glucose simultaneously; glucose outcompetes xylose for uptake into the cell *via* the endogenous hexose transport system of yeast (Kötter & Ciriacy, 1993; W. J. Lee, et al., 2002).

However, cellodextrin transport represents an alternate and highly selective route for glucose to enter yeast. Working with the groups of Yong-Su Jin and Huimin Zhao at the University of Illinois Urbana-Champaign, we demonstrated that a cellodextrin transport pathway allowed xylose-fermenting strains of *S. cerevisiae* to coferment xylose and glucose (in the form of cellobiose) simultaneously and synergistically, with an increase in both ethanol yield and productivity (Ha, et al., 2011; Li, et al., 2010).

We are just beginning to understand the mechanisms of biomass degradation by *N. crassa*, and expect that many interesting and consequential stories remain to be told. Ultimately we hope to build a complete description of this process including: (i) how *N. crassa* detects and responds to the presence of plant cell wall material; (ii) how this material is enzymatically depolymerized to soluble components; (iii) and how these components are subsequently metabolized. We believe this effort will continue to yield ideas and technologies of use in the production of lignocellulosic biofuels, and our collaborations with bioengineers and synthetic biologist will help to implement these. While our goals are ambitious, we are optimistic that through the combined efforts of diverse scientists and engineers, we will succeed in making economical and sustainable biofuel production a reality.

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Figures and Tables

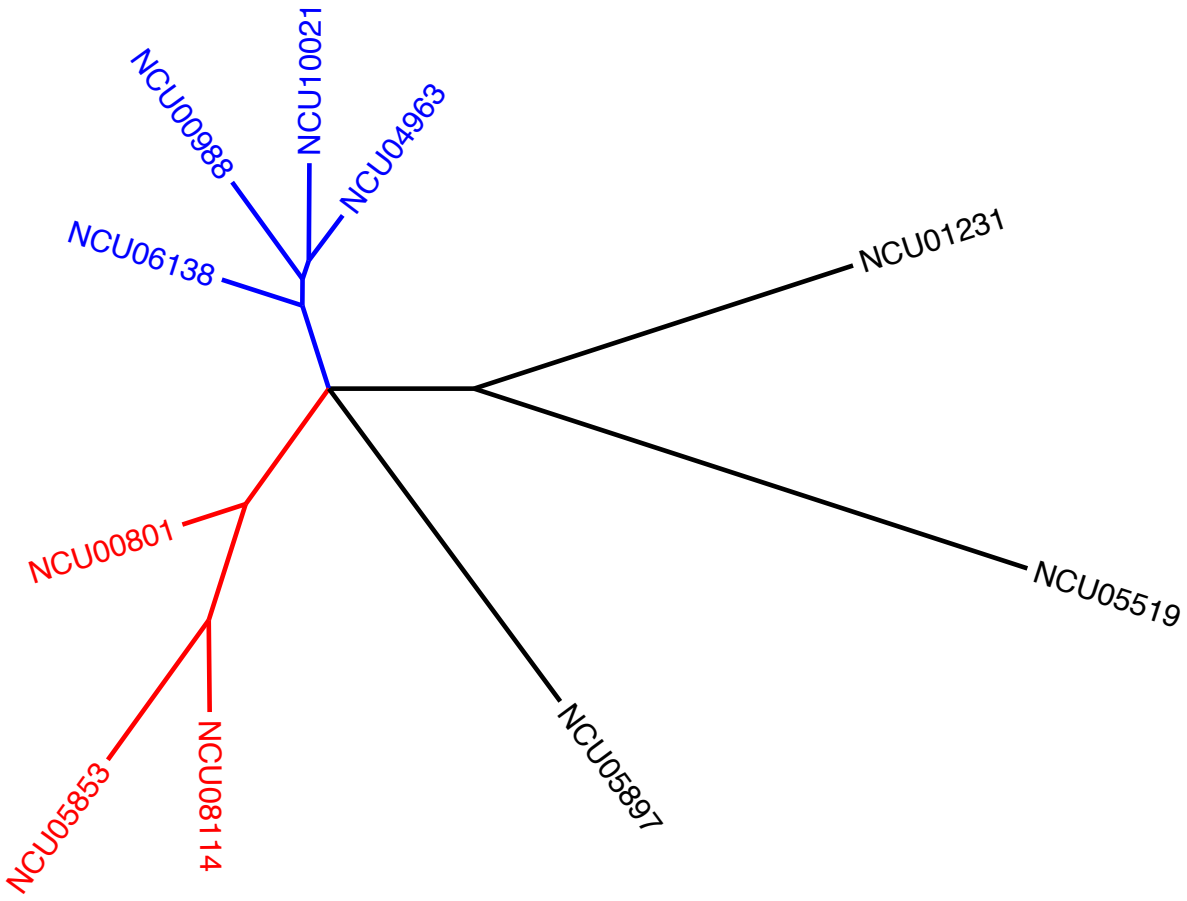

Figure 2-1. An unrooted phylogram of the 10 major facilitator superfamily transporters induced by *N. crassa* in response to cellulose. Amino acid sequences were aligned with T-Coffee. This alignment was curated with Gblocks, and the output used to build a phylogenetic tree by the maximum likelihood method implemented in PhyML. The tree was visualized with FigTree.

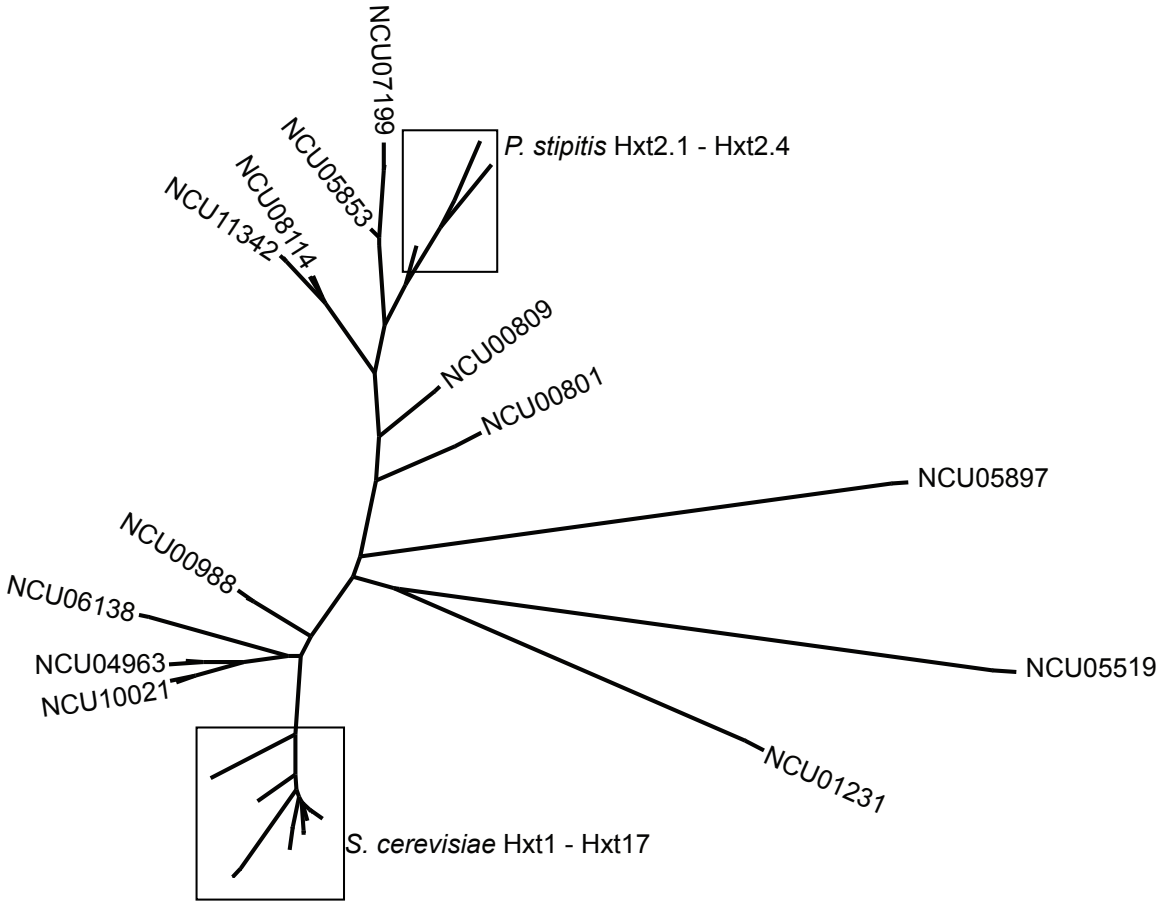

Figure 2-2. An unrooted phylogram showing the relationship of the 10 cellulose-induced transporters to other *N. crassa* transporters, their orthologs in *C. globosum*, and the *S. cerevisiae* and *P. stipitis* hexose transporters. The amino acid sequence of the indicated *N. crassa* genes, their closest homolog in *C. globosum*, the *S. cerevisiae* hexose transporters (Hxt1 – Hxt17), and the *P. stipitis* hexose transporters (Hxt2.1 - Hxt2.4) were aligned with MUSCLE. This alignment was curated with Gblocks, and the output used to build a phylogenetic tree by the maximum likelihood method implemented in PhyML. The tree was visualized with FigTree. *N. crassa* genes and their closest *C. globosum* homologs cluster very closely, and the *C. globosum* gene names are left out for clarity. The *S. cerevisiae* and *P. stipitis* hexose transporters cluster with themselves, thus individual gene names are left out for clarity.

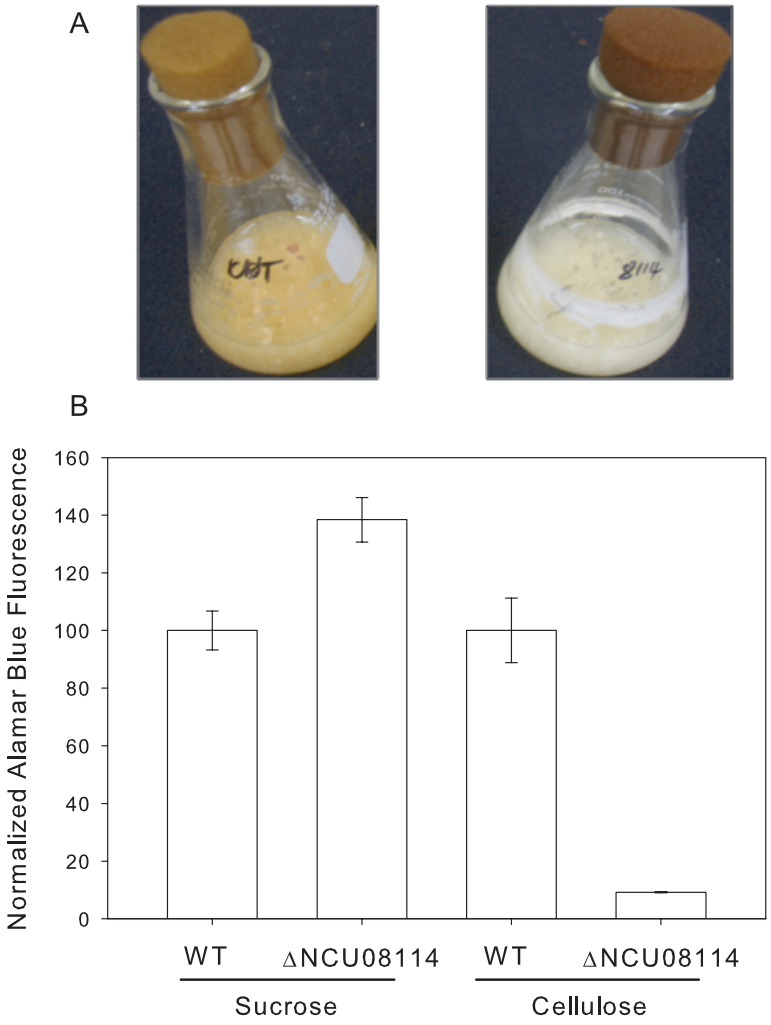

Figure 2-3. Growth phenotype of a *N. crassa* strain lacking NCU08114. (A) Shaker flasks of WT (left) and ΔNCU08114 (right) *N. crassa* strains after 3 days of growth with crystalline cellulose as a carbon source. **(B)** Alamar Blue© fluorescence from *N. crassa* cultures grown with either sucrose or crystalline cellulose as a carbon source for 16 or 28 hours, respectively (average of three biological replicates ±SD). Fluorescence was normalized by setting WT to 100%. *N. crassa* lacking NCU00801 did not have an obvious phenotype (not shown).

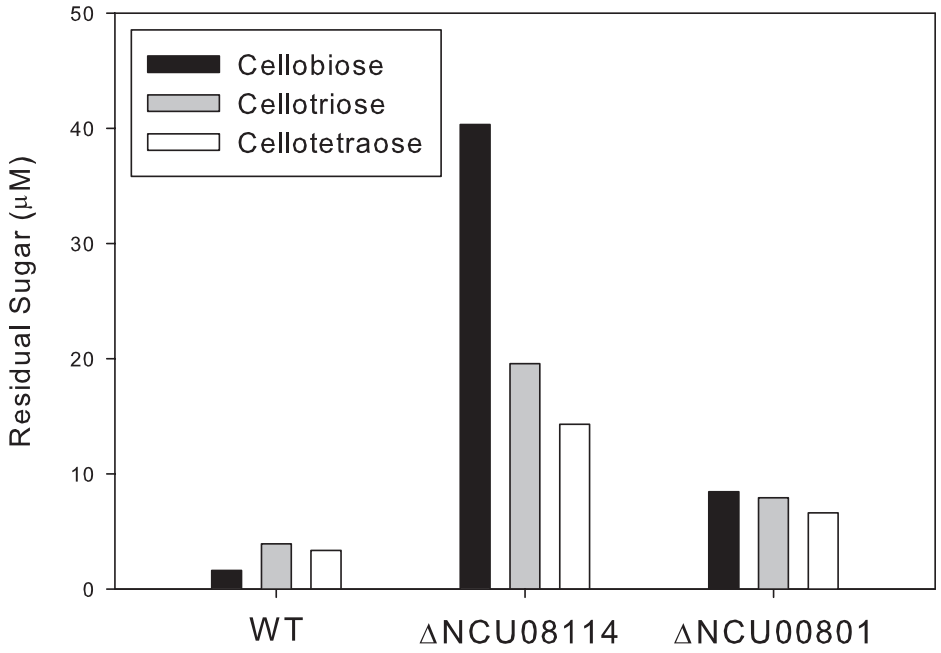

Figure 2-4. Cellodextrin consumption by *N. crassa* strains lacking NCU00801 or NCU08114. The indicated *N. crassa* strains were incubated with 90 μM of the respective sugars for 15 minutes. Bars represent the mean concentration of sugars remaining in the supernatant following the incubation from two independent experiments. *N. crassa* secretes β-glucosidases that hydrolyze cellodextrins to glucose, which is subsequently taken up by monosaccharide transporters. This alternate route of consumption leads to an underestimate of the cellodextrin transport defect in these deletion lines.

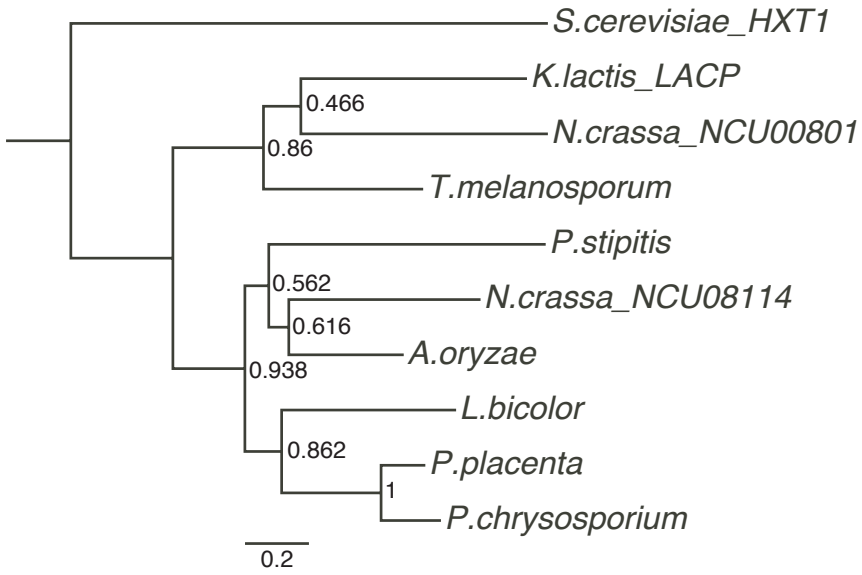

Figure 2-5. Maximum likelihood phylogenetic analysis of the cellobiose transporters NCU08114 and NCU00801. With the exception of *S. cerevisiae* HXT1 and *K. lactis* LACP, all genes encoding proteins shown are reported to increase in expression level when the fungus comes into contact with plant cell wall material or cellobiose (Martin, et al., 2010; Noguchi, et al., 2009; Tian, et al., 2009; Vanden Wymelenberg, et al., 2010). *S. cerevisiae* HXT1, a low affinity glucose transporter (Reifenberger, et al., 1997), was used as an outgroup. GenBank accession numbers or Joint Genome Institute (JGI) protein ID (PID) numbers for cellodextrin transporters are as follows: *Tuber melanosporum*, CAZ81962.1; *Pichia stipitis*, ABN65648.2; *Laccaria bicolor*, EDR07962, *Aspergillus oryzae*, BAE58341.1; *Phanerochaete chrysosporium*, PID 136620 (JGI) (S1); *Postia placenta*, PID 115604 (JGI) (S2). The GenBank accession number for *Saccharomyces cerevisiae* HXT1 and *Kluyveromyces lactis* LACP are DAA06789.1 and CAA30053.1, respectively. The *P. chrysosporium* and *P. placenta* genomes can be accessed at http://genome.jgi-psf.org/Phchr1/Phchr1.home.html and http://genome.jgi-psf.org/Pospl1/Pospl1.home.html, respectively.

	GH family	GenBank accession #
T. melanosporum	GH1	CAZ82985
A. oryzae	GH1	BAE57671
P. placenta	GH1	EED81359
P. chrysosporium	GH1	BAE87009
K. lactis	GH3	CAG99696
L. bicolor	GH3	EDR09330
C. lusitaniae	GH3	EEQ37997
P. stipitis	GH3	ABN67130

Table 2-6. GenBank accession numbers for predicted intracellular cellodextrin hydrolases in fungi that grow on cellulose or cellodextrins. The indicated fungal species grow upon or degrade plant cell walls, or metabolize cellodextrins. They are predicted to express an intracellular β-glucosidase from the indicated Glycoside Hydrolase (GH) family with the indicated GenBank accession number.

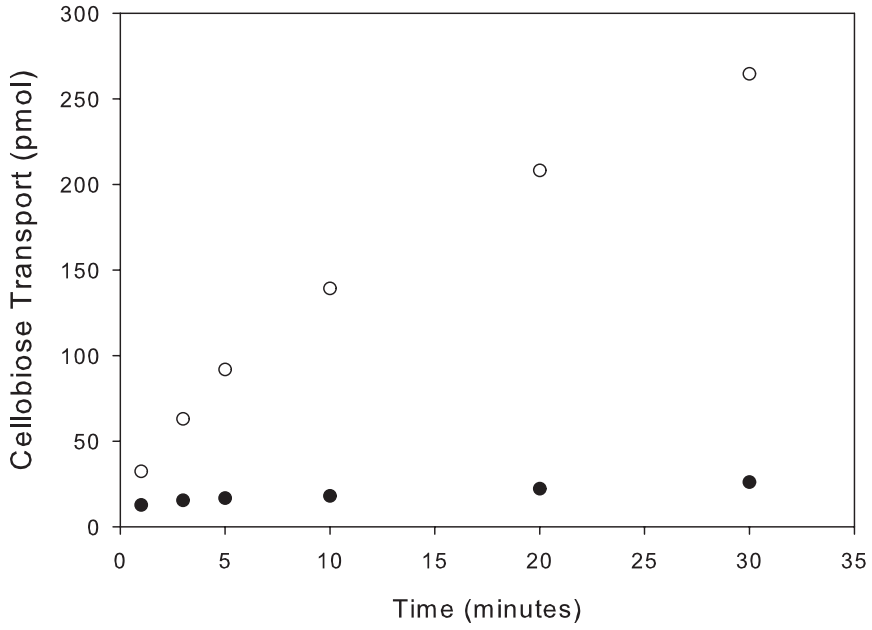

Figure 2-7. Cellobiose transport by a *S. cerevisiae* strain expressing *cdt-1.* Shown is cellobiose transport by yeast with (○) or without (●) CDT-1. Both strains express the intracellular β-glucosidase, NCU00130 (named GH1-1). The initial concentration of cellobiose was 50 μM. All values are the mean between two measurements.

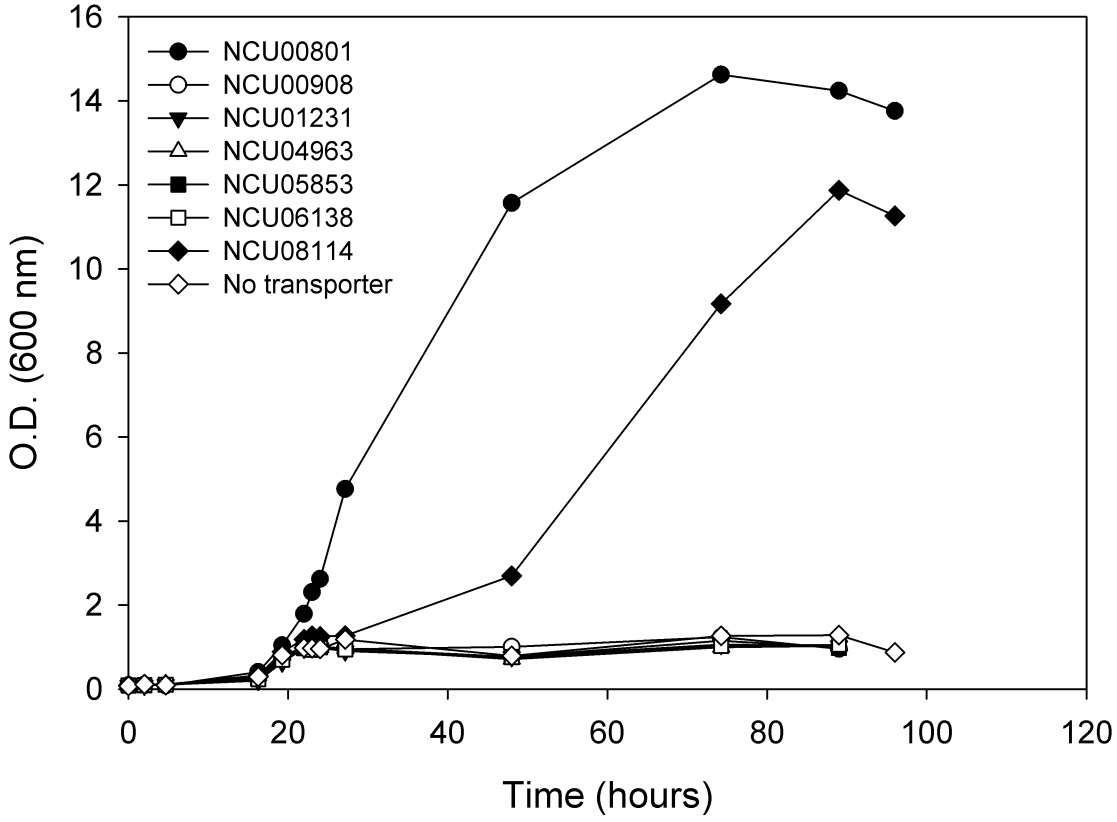

Figure 2-8. Growth of *S. cerevisiae* strains transformed with one *N. crassa* transporter and an intracellular β-glucosidase from *N. crassa*. An *S. cerevisae* strain expressing the intracellular β-glucosidase, *gh1-1* (NCU00130), was transformed with a plasmid containing one of the indicated *N. crassa* transporters, or an empty plasmid (No transporter). After pre-growing these strains on glucose, the growth rate of the strains was determined with 2% cellobiose as the sole carbon source.

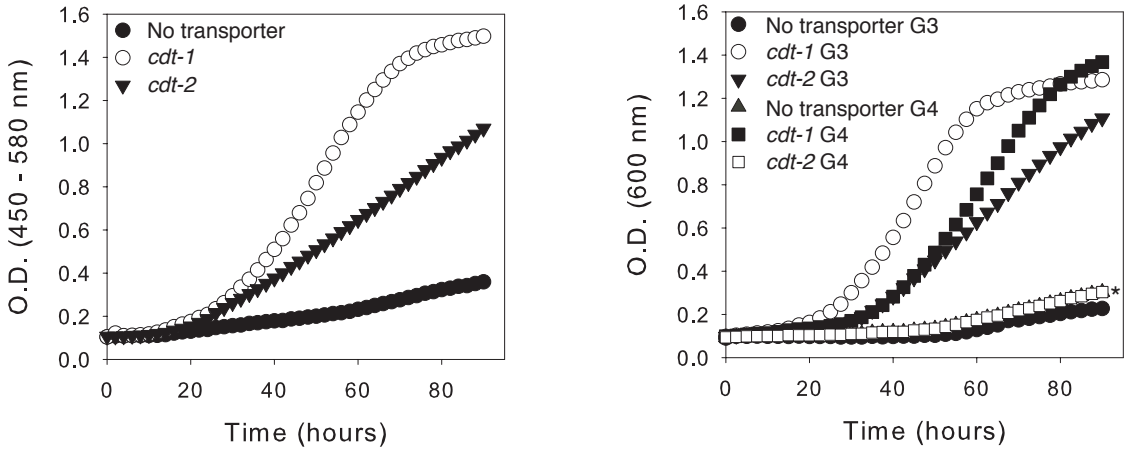

Figure 2-9. Cellodextrin-mediated growth of *S. cerevisiae* strains expressing a cellodextrin transport pathway. (Left Panel) Cellobiose-mediated growth of yeast strains expressing the gene NCU00801 (named *cdt-1*, ○), NCU08114 (named *cdt-2*, ▼), or no transporter (●). All strains also express the intracellular β-glucosidase, NCU00130 (named *gh1-1*). A representative experiment is shown. **(Right Panel)** Growth of yeast strains on cellotriose and cellotetraose. Strains expressing the intracellular β-glucosidase, *gh1-1*, as well as the transporters listed in the legend, were grown with 0.5% (w/v) of cellotriose (G3) or cellotetraose (G4) serving as the sole carbon source. A representative experiment is shown. Note that the data for yeast growth on cellotetraose with *cdt-2* overlaps the data in the absence of transporter.

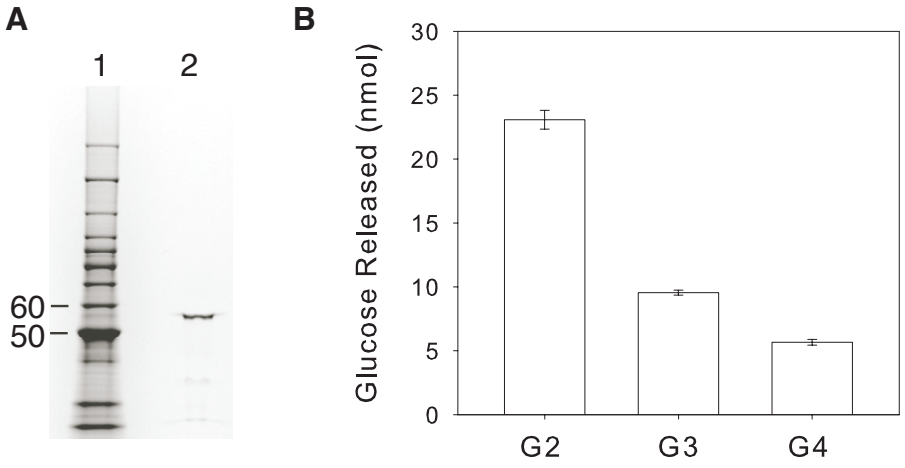

Figure 2-10. Hydrolysis of cellodextrins by NCU00130 (GH1-1). (A) SDS-PAGE gel of purified GH1-1. Lane 1, Protein molecular weight standards, in kDa. Lane 2, GH1-1 after purification over nickel-NTA resin. Molecular weights in kDa are shown to the left. **(B)** Glucose produced from cellobiose (G2), cellotriose (G3), and cellotetraose (G4) hydrolysis by purified GH1-1 (average of three independent experiments ±SD). Residual glucose in incubations without enzyme (2 nmol) was subtracted from the values shown.

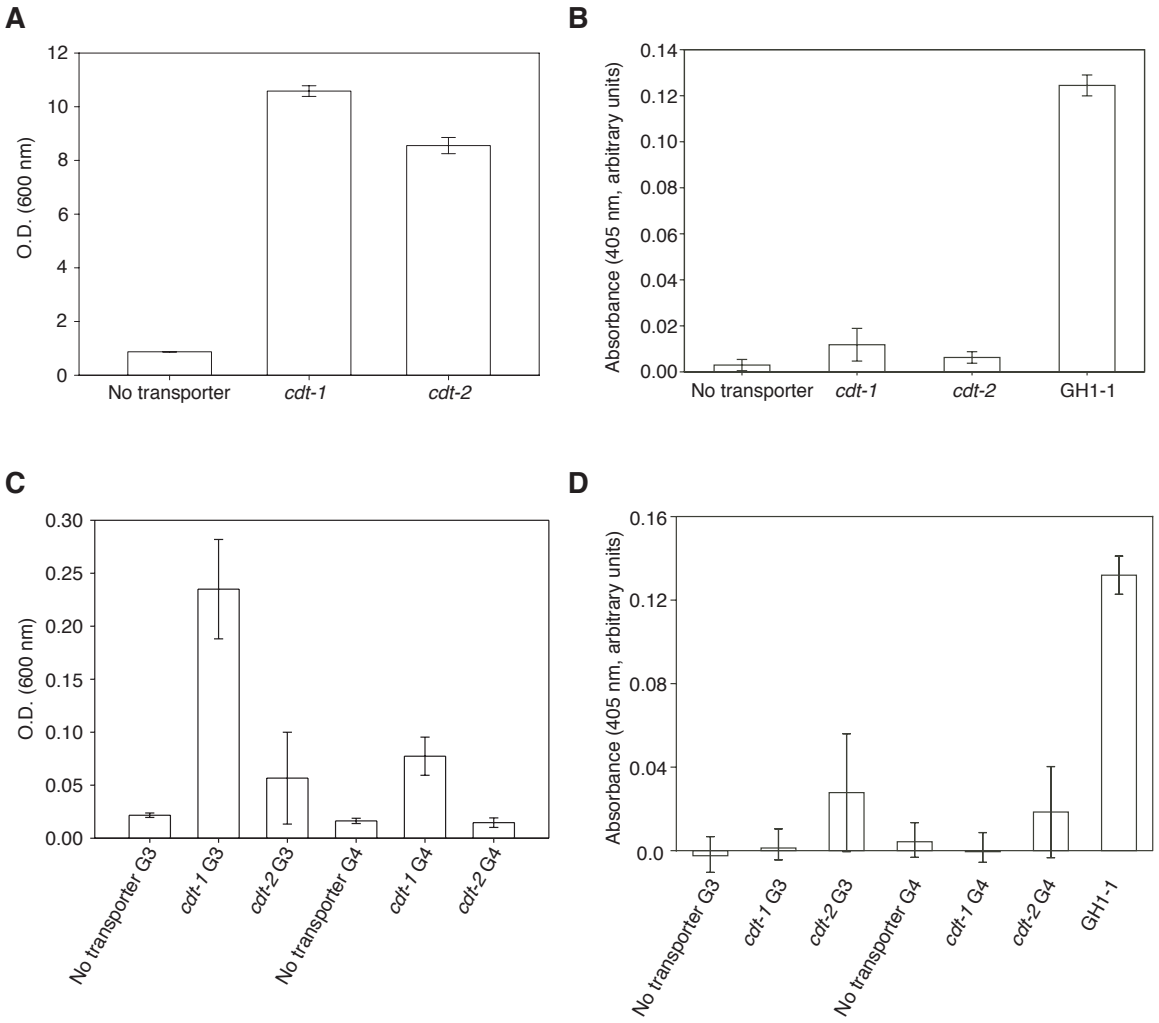

Figure 2-11. β-glucosidase activity in cell culture broth after growth experiments. (A) Final OD of yeast cultures grown in 250 mL shake flasks for 84 hours (mean of three technical replicates ±SD) with cellobiose as a sole carbon source. **(B)** β-glucosidase activity measured by *p*-Nitrophenol generation (mean of three technical replicates ± SD). Filtered culture broths from the cultures in (A) were used. Purified β-glucosidase GH1-1 (6.3 nM) was used as a positive control. **(C)** Final OD of yeast cultures grown in a Bioscreen C™ for 96 hours (mean of three biological replicates ± SD) with cellotriose or cellotetraose as a sole carbon source. **(D)** β-glucosidase activity measured by *p*-Nitrophenol generation (mean of three biological replicates ± SD). Filtered culture broths from the cultures in (C) were used. Purified β-glucosidase GH1-1 (6.3 nM) was used as a positive control.

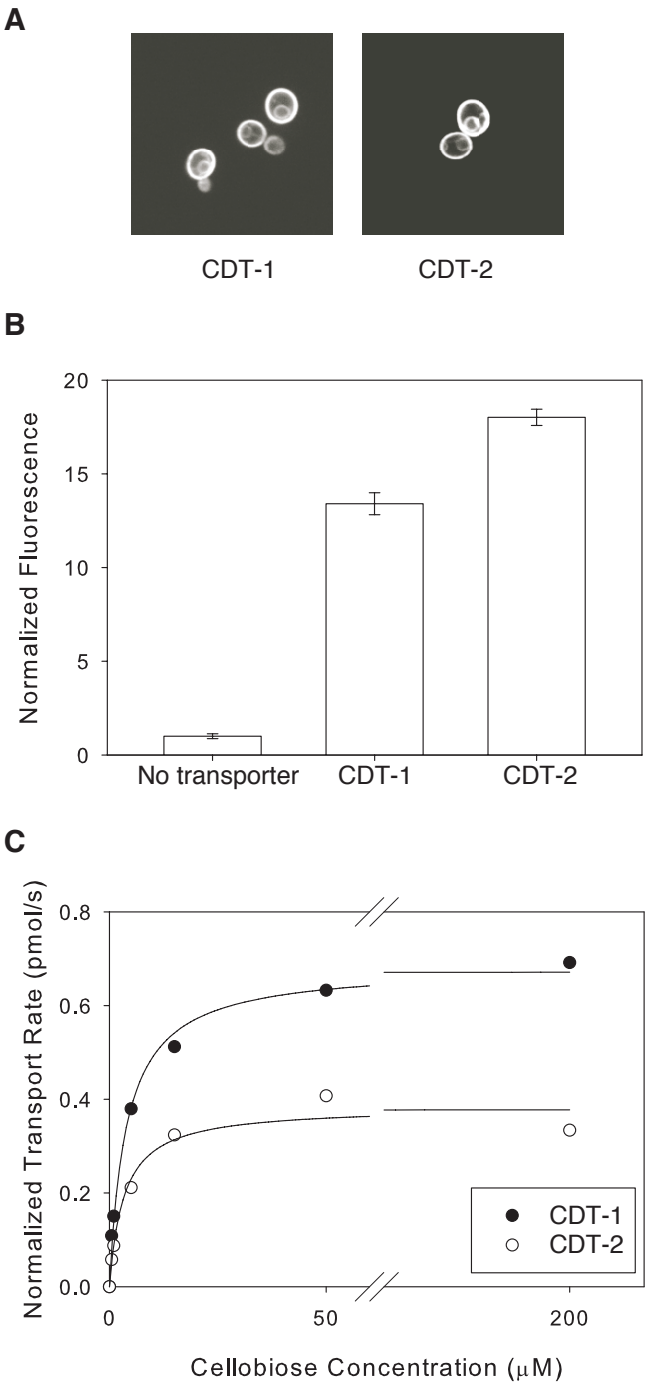

Figure 2-12. Kinetics of cellobiose transport by CDT-1 and CDT-2. (A) Localization of GFP fused to CDT-1 and CDT-2. Images of *S. cerevisiae* strains expressing *cdt-1* (left), or *cdt-2* (right), fused to GFP at their C-terminus. **(B)** GFP fluorescence of yeast strains without a cellobiose transporter, or expressing *cdt-1* or *cdt-2* fused to GFP at their C-terminus (average of three biological replicates ± SD). **(C)** The rate of cellobiose transport was determined as a function of cellobiose concentration by yeast strains expressing either *cdt-1* or *cdt-2.* The transport rate was normalized for transporter abundance.

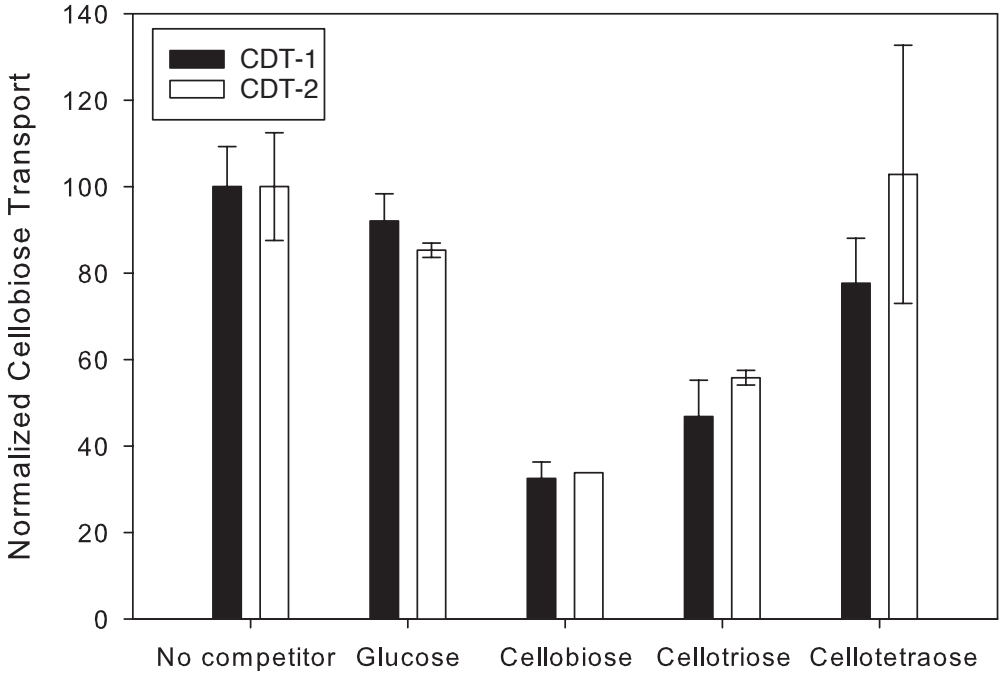

Figure 2-13. Competition by cellodextrins for cellobiose transport in strains expressing *cdt-1* or *cdt-2.* A 5-fold excess of the respective unlabeled sugar was included during assays of [^{3}H]-cellobiose transport. Substrates of CDT-1 or CDT- 2 should decreases the [^{3}H]-cellobiose transport rate by competing for binding. Values (average of three independent experiments ±SD) were normalized by setting the rate of [^{3}H]-cellobiose transport without a competing sugar to 100.

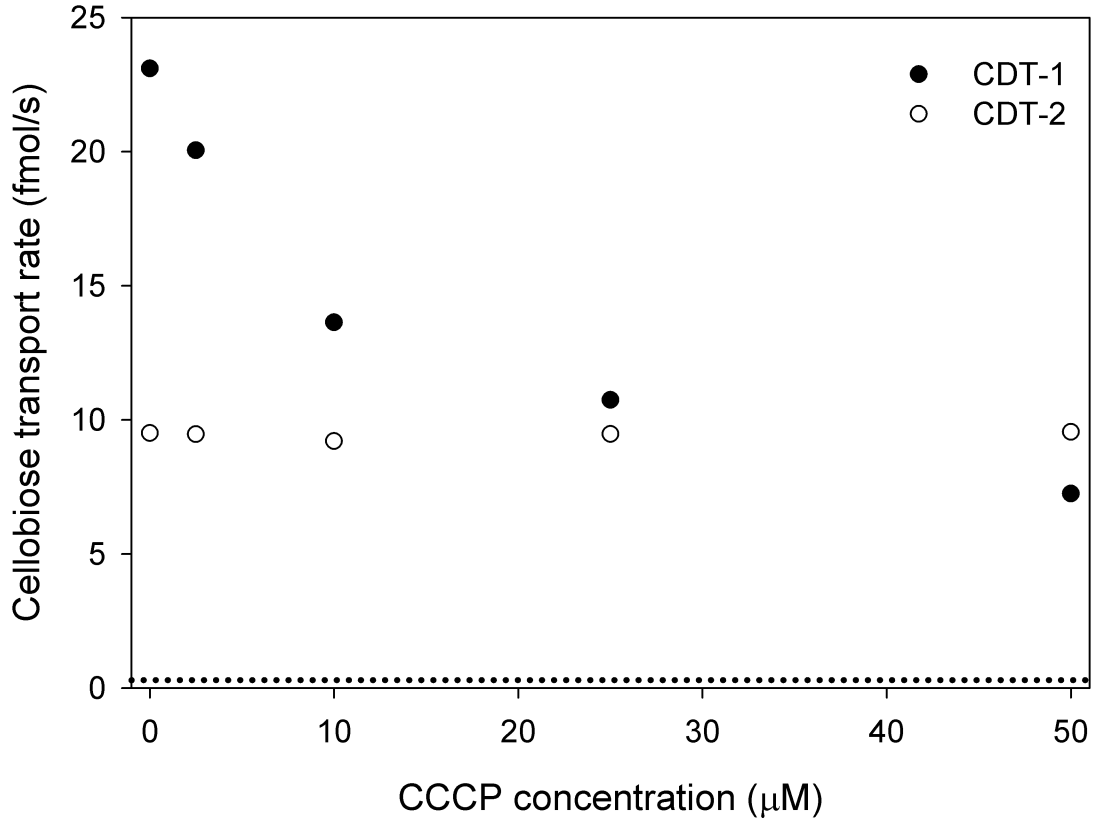

Figure 2-14. Senstivity of cellobiose transport by CDT-1 and CDT-2 to the proton uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). *S. cerevisiae* strains expressing *cdt-1* (●) or *cdt-2* (○) were incubated with the indicated concentration of CCCP for 10 minutes at room temperature. Then the rate at which these cells imported 0.25 μM [^{3}H]-cellobiose was measured. The dashed line indicates the rate of transport by S. cerevisiae without *cdt-1* or *cdt-2*.

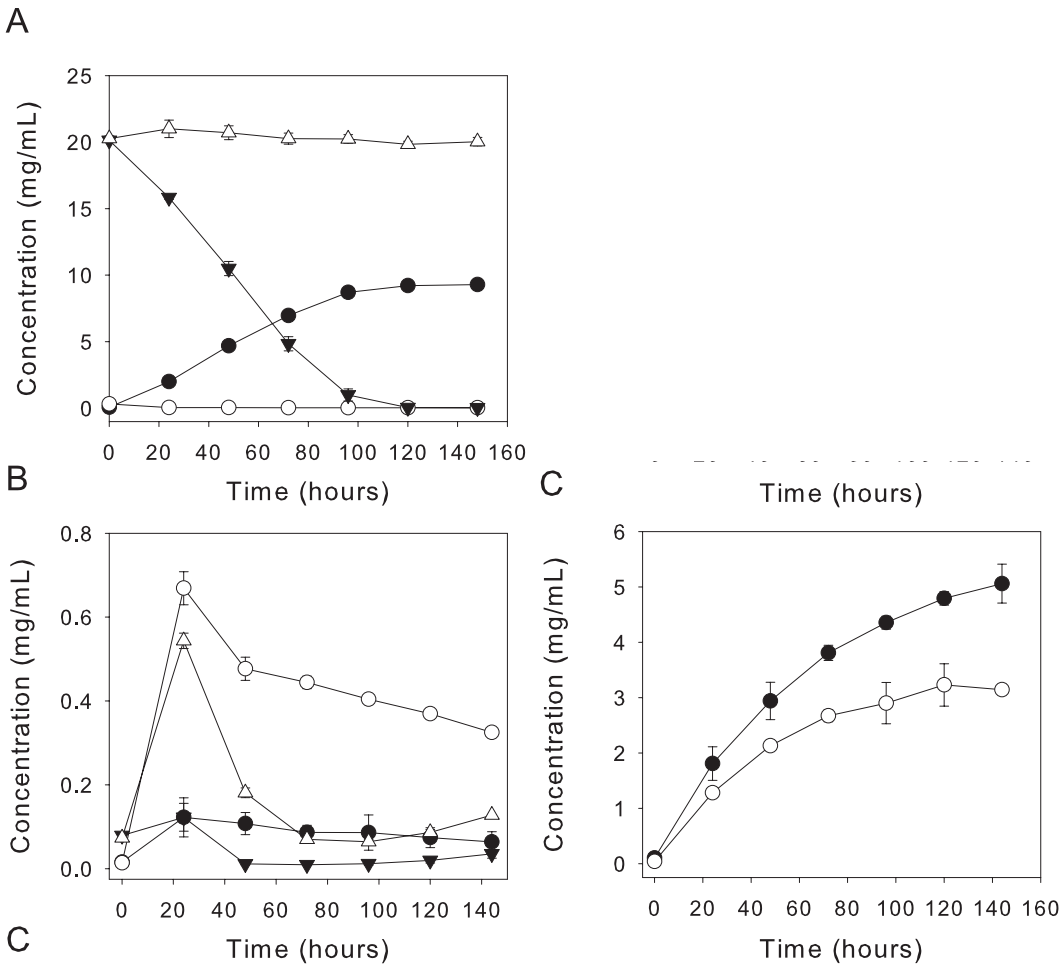

Figure 2-15. Cellobiose fermentation, and simultaneous saccharification and fermentation of cellulose, by *S. cerevisiae* expressing the cellobiose transport system from *N. crassa*. (A) Cellobiose fermentation to ethanol**.** Ethanol produced by yeast strains with CDT-1 (●), or without CDT-1 (○) under anaerobic conditions. Cellobiose concentration during the fermentation reaction using yeast strains with CDT-1 (▼), or without CDT-1 (△). **(B)** SSF using yeast strains with and without CDT-1, under anaerobic conditions. Cellobiose (●) and glucose (▼) concentrations in the presence of a strain with CDT-1, and cellobiose (○) and glucose (△) concentrations in the presence of a strain lacking CDT-1. Note, 0.1 mg/mL cellobiose = 292 µM. **(C)** Ethanol produced during SSF using a strain with CDT-1 (●), or without CDT-1 (○). In all panels, values are the mean of 3 biological replicates. Error bars are the standard deviation between these replicates. All strains also express the intracellular β-glucosidase, *gh1-1*.

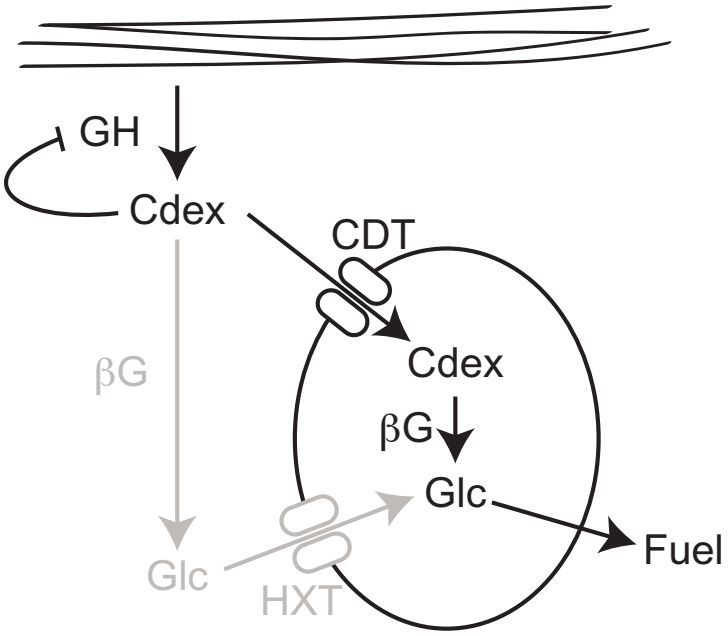

Figure 2-16. Use of cellodextrin transport pathways from filamentous fungi in yeast during simultaneous saccharification and fermentation of cellulose. The cellodextrin (Cdex) transport pathway (black) includes a cellodextrin transporter (CDT) and intracellular β-glucosidase (βG). The sugar catabolism pathway present in standard yeast includes hexose transporters (HXT). In SSF, cellulases (GH) and extracellular β-glucosidase (βG) may both be used.

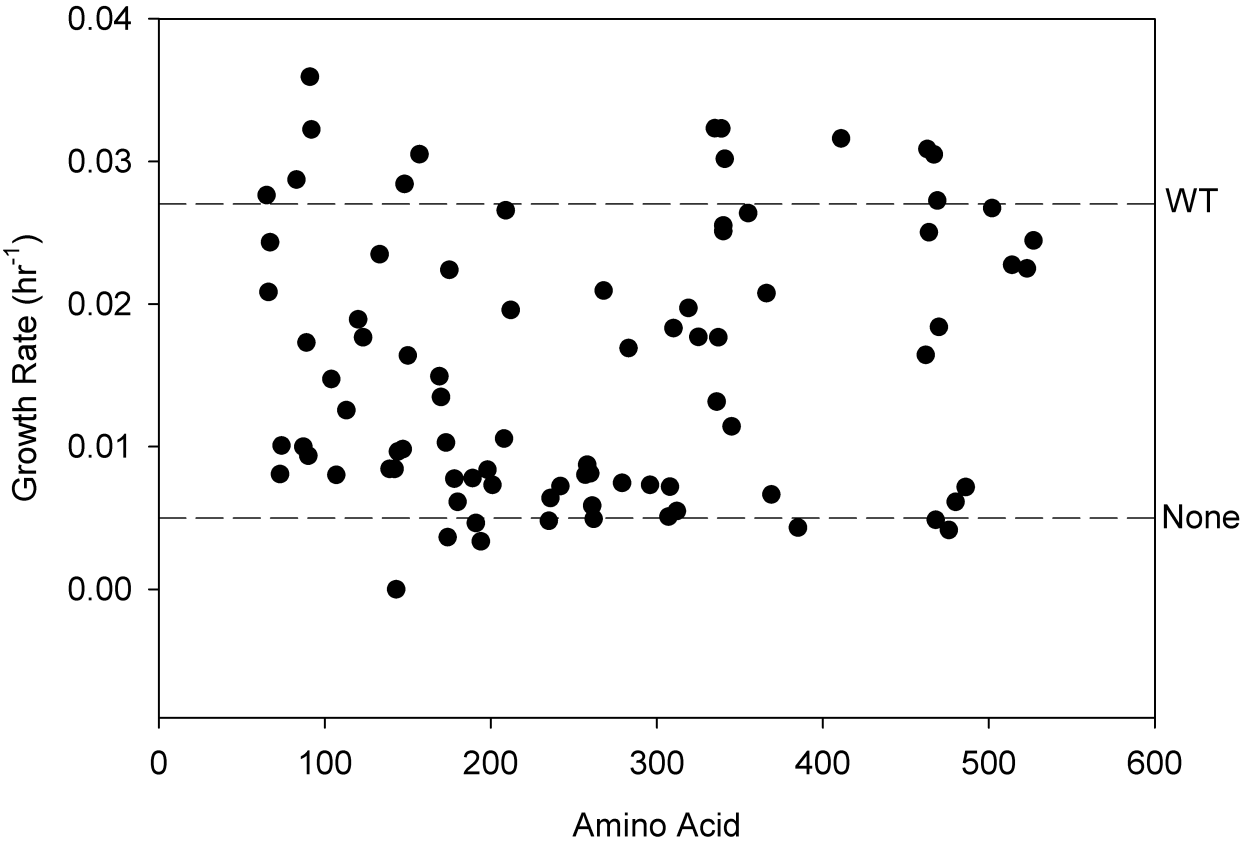

Figure 3-1. Cellobiose-mediated growth rates of *S. cerevisiae* transformed with various CDT-1 alanine mutants and GH1-1. Conserved amino acids in CDT-1 were separately mutated to alanine. A CDT-1 construct containing a mutation at one positions was transformed into *S. cervisiae* along with the β-glucosdiase, GH1-1. These strains were grown with cellobiose as a sole carbon source. The growth rates (increase in the OD at 600 nm per hour) were determined and compared to strains with WT CDT-1 and GH1-1 or no transporter and GH1-1. Each point represents the average of two measurements. The position of a point on the x-axis corresponds to its position in the primary sequence of CDT-1 e.g. the initiating methionine would have an x-value of 1.

Mutated Residue	Growth Rate (%WT)	Mutated Residue	Growth Rate (%WT)	Mutated Residue	Growth Rate (%WT)
S67	88.6	L179	*n.d.*	F335	117.8
L73	29.4	G180	22.3	G336	47.9
Y74	36.7	P189	28.4	Q337	64.4
C83	104.6	Y191	16.9	S339	117.7
N87	36.4	E194	12.2	G340	92.9
G88	*n.d.*	P198	30.5	N341	110.0
Y89	63.0	R201	26.7	Y345	41.6
D90	34.1	Y208	38.5	G355	96.1
G91	130.9	N209	96.8	N366	75.6
S92	117.5	W212	71.4	N369	24.2
P65	100.7	G215	*n.d.*	D385	15.7
W66	75.9	W235	17.5	R389	*n.d.*
Q104	53.7	R236	23.3	R340	91.5
F107	29.2	Q242	26.4	S411	115.2
G113	45.7	P257	29.3	F462	59.9
F120	68.9	E258	31.8	S463	112.5
Y123	64.4	S259	29.6	F464	91.2
G126	*n.d.*	P260	29.6	T467	111.1
F133	85.6	R261	21.4	P468	17.7
D139	30.7	F262	18.0	L469	99.3
G142	30.7	R268	76.3	Q470	67.0
R143	*n.d.*	Y279	27.2	E476	15.1
K144	35.2	H280	*n.d.*	T480	22.3
M147	35.8	G283	61.6	R483	*n.d.*
F148	103.5	E296	26.7	K485	*n.d.*
G150	59.7	D307	18.6	G486	26.1
G157	111.1	K308	26.2	Q502	97.3
Q169	54.4	W310	66.7	Y514	82.8
F170	49.1	D312	20.0	W523	81.9
G173	37.4	Y313	*n.d.*	E527	89.1
R174	13.3	T319	71.8	WT	100.0
F175	81.6	R323	*n.d.*	None	22.0
G178	28.3	R325	64.5		

Table 3-2. Cellobiose-mediated growth rates of *S. cerevisiae* transformed with various CDT-1 alanine mutants and GH1-1. Conserved amino acids in CDT-1 were separately mutated to alanine. A CDT-1 construct containing a mutation at one position was transformed into *S. cerevisiae* along with the β-glucosidase, GH1-1. These strains were grown with cellobiose as a sole carbon source. The growth rates (increase in the OD at 600 nm per hour) were determined and compared to strains with WT CDT-1 and GH1-1 or no transporter and GH1-1. Each value represents the average of two measurements, and is reported as a percentage of the WT growth rate. *n.d.*= not determined.

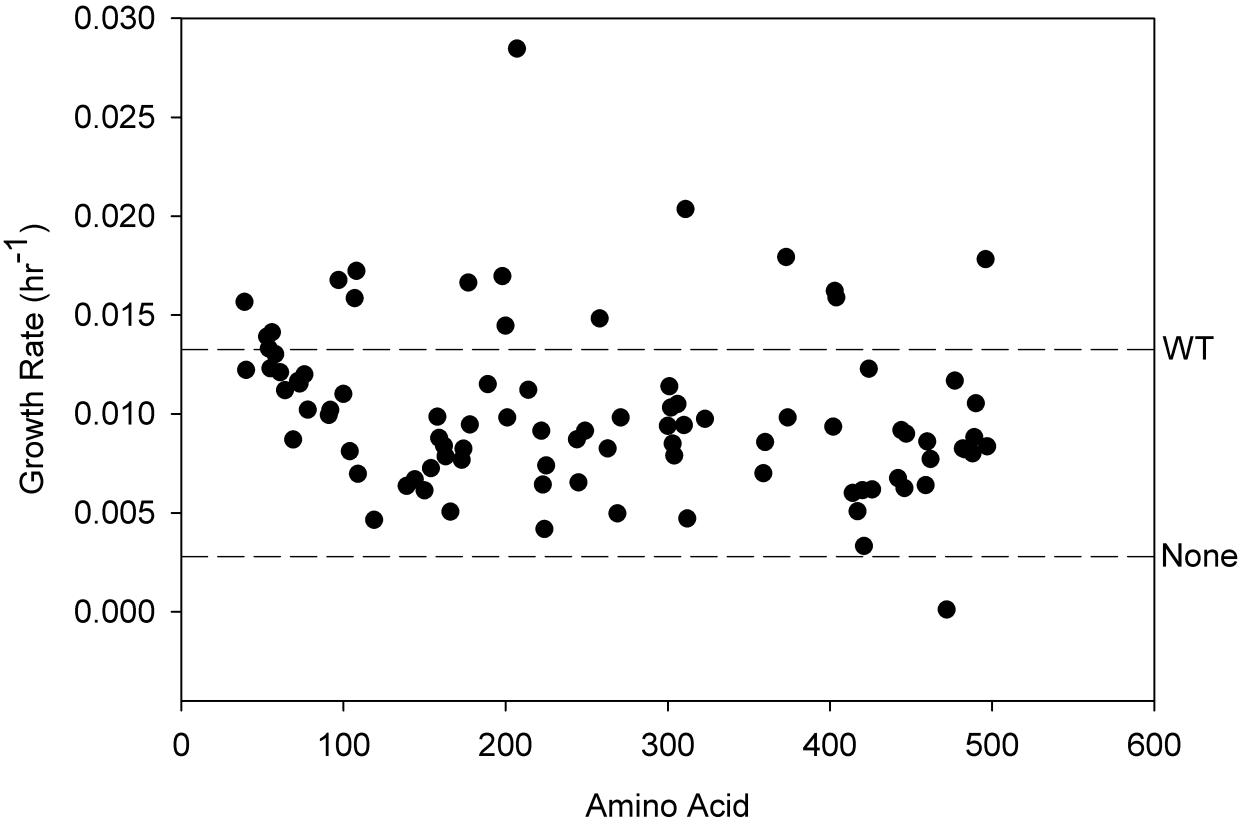

Figure 3-3. Cellobiose-mediated growth rates of *S. cerevisiae* transformed with various CDT-2 alanine mutants and GH1-1. Conserved amino acids in CDT-2 were separately mutated to alanine. A CDT-2 construct containing a mutation at one positions was transformed into *S. cerevisiae* along with the β-glucosidase, GH1-1. These strains were grown with cellobiose as a sole carbon source. The growth rates (increase in the OD at 600 nm per hour) were determined and compared to strains with WT CDT-2 and GH1-1 or no transporter and GH1-1. Each point represents the average of two measurements. The position of a point on the x-axis corresponds to its position in the primary sequence of CDT-2 e.g. the initiating methionine would have an x-value of 1.

Mutated Residue	Growth Rate (%WT)	Mutated Residue	Growth Rate (%WT)	Mutated Residue	Growth Rate (%WT)
L39	119.6	S224	31.9	K426	47.2
Y40	93.3	P225	56.4	N442	51.5
T53	106.2	R226	*n.d.*	Y444	70.1
G54	101.6	Y244	66.5	N446	47.7
Y55	93.9	H245	49.9	P447	68.7
D56	107.9	D249	69.8	W459	48.8
G57	*n.d.*	E258	113.1	K460	65.7
M58	99.4	E261	*n.d.*	Y462	58.9
N61	92.4	K263	63.0	E472	*n.d.*
Q64	85.5	E269	37.9	E482	63.0
W69	66.5	E271	75.0	T483	62.7
Y72	89.0	F300	71.7	L488	61.1
F73	88.0	S301	87.0	E489	67.3
P76	91.6	Q302	78.8	E490	80.4
G91	75.9	W303	64.9	D496	136.0
S92	77.9	S304	60.3	G497	63.8
P97	128.0	N306	80.2	G78	78.0
P100	84.1	S310	72.0	R139	48.5
D104	61.9	N311	155.4	P154	55.3
G107	121.0	Y312	36.0	T158	75.2
R108	131.5	T323	74.4	E159	67.1
K109	53.2	F359	53.4	H162	64.1
M119	35.4	L360	65.4	N178	72.2
F144	51.1	W373	136.9	F189	87.8
Q150	46.8	T374	75.0	W198	129.5
P163	60.0	F402	71.4	W200	110.4
R166	38.6	Y403	123.8	R201	75.0
T170	*n.d.*	S404	121.3	Q207	217.3
Y173	58.6	Y414	45.8	Q214	85.6
N174	62.9	E417	38.7	F477	89.1
W177	127.0	P420	46.9	WT	100.0
P222	69.8	Y421	25.4	None	22.9
E223	49.1	R424	93.8		

Table 3-4. Cellobiose-mediated growth rates of *S. cerevisiae* transformed with various CDT-2 alanine mutants and GH1-1. Conserved amino acids in CDT-2 were separately mutated to alanine. A CDT-2 construct containing a mutation at one position was transformed into *S. cerevisiae* along with the β-glucosidase, GH1-1. These strains were grown with cellobiose as a sole carbon source. The growth rates (increase in the OD at 600 nm per hour) were determined and compared to strains with WT CDT-2 and GH1-1 or no transporter and GH1-1. Each value represents the average of two measurements, and is reported as a percentage of the WT growth rate. *n.d.* = not determined.

N. crassa ortholog	Species	Identifier*	Growth Rate (%WT)	Growth Rate Error (St.Dev.)
CDT-1	*N. crassa*	XP_963801.1	100.0	7.3
	No transporter		5.3	0.2
CDT-1	*A. nidulans*	XP_660418.1	1.8	0.9
CDT-1	*M. grisea*	XP_364883.1	10.4	2.8
CDT-1	*A. fumigatus*	XP_753099.1	8.2	4.3
CDT-1	*T. atroviridae*	211304	5.5	0.6
CDT-1	*C. globosum*	XP_001220469.1	12.1	0.2
CDT-1	*T. mesenterica*	63529	9.0	2.4
CDT-1	*H. annosum*	105952	5.6	1.4
CDT-1	*C. parasitica*	252427	46.2	0.4
CDT-1	*T. reesei*	67752	7.5	1.4
CDT-1	*A. clavatus*	XP_001268541.1	29.5	1.2
CDT-1	*N. discreta*	77429	14.5	2.3
CDT-1	*T. reesei*	3405	6.7	2.1
CDT-1	*S. thermophile*	43941	28.0	8.9
CDT-2	*T. atroviridae*	215408	1.7	1.3
CDT-2	*C. globosum*	XP_001220290.1	8.3	1.0
CDT-2	*A. nidulans*	ANID_08347	22.8	0.1
CDT-2	*P. ostreatus*	61138	8.0	0.4
CDT-2	*S. thermophile*	114107	19.6	4.4
NCU05853	*C. globosum*	XP_001226269.1	7.4	1.3
NCU05853	*T. reesei*	46819	4.2	0.2
NCU05853	*M. graminicola*	68287	6.0	1.2
NCU05853	*A. flavus*	AFLA_000820A	6.3	4.3

Table 3-5. Growth rate of *S. cerevisiae* strains expressing various CDT-1, CDT-2 or NCU05853 orthologs, along with GH1-1. A construct containing the indicated gene was transformed into *S. cerevisiae* along with the β-glucosidase, GH1-1. These strains were grown with cellobiose as a sole carbon source. The growth rates were determined and compared to strains with WT CDT-1 and GH1-1 or no transporter and GH1-1. Each value represents the mean of three measurements, and is reported as a percentage of the WT growth rate. The reported error is the standard deviation between these measurements.

*Accession numbers are used to identify the genes unless these are not available. In this case, the JGI number was used. This number allows access to the gene sequence via the JGI genome portal for this organism (accessible from the following page: http://genome.jgi-psf.org/programs/fungi/index.jsf). The *A. nidulans* and *A. flavus* identifiers allow access to the genes through their genome portals at http://www.aspergillusgenome.org/ and http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html, respectively.

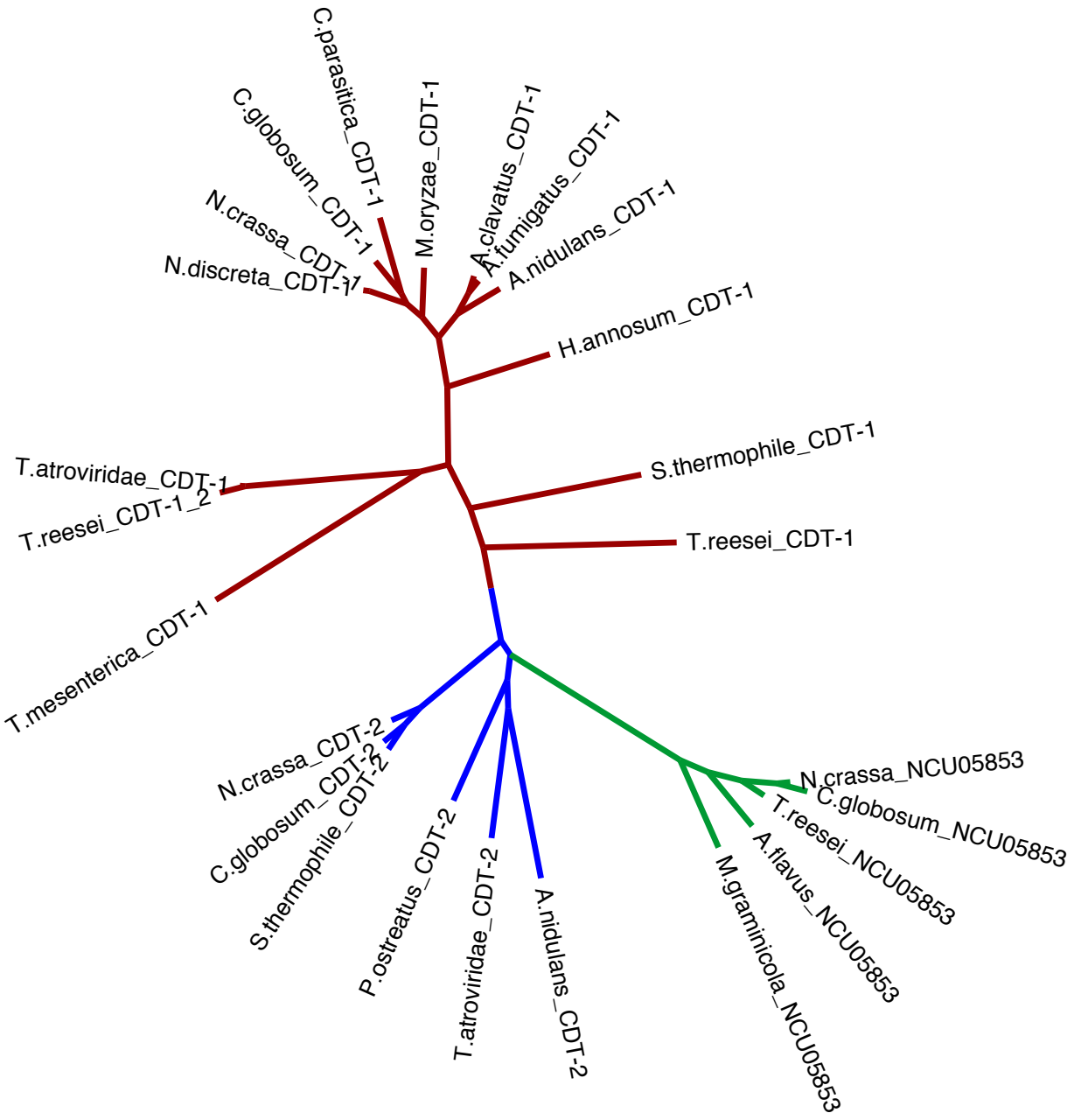

Figure 3-6. Maximum likelihood phylogenetic tree of tested orthologs of CDT-1, CDT-2 and NCU05853. Amino acid sequences were aligned with MUSCLE, alignments curated with Gblocks, and a phylogenetic tree reconstructed using the maximum likelihood method implemented in PhyML. The tree was visualized with FigTree.

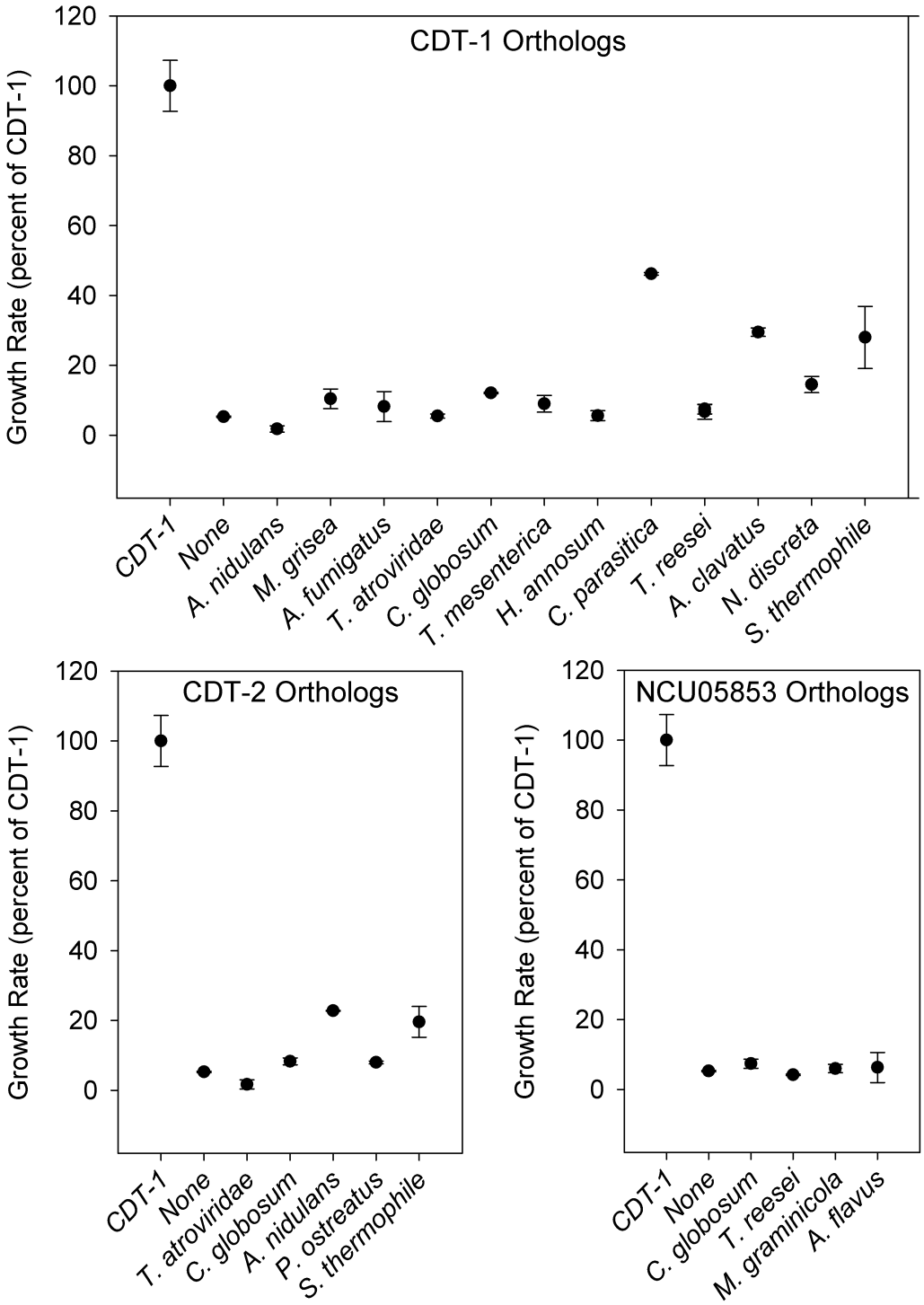

Figure 3-7. Growth rate of *S. cerevisiae* strains expressing various CDT-1, CDT-2 or NCU05853 orthologs, along with GH1-1. A construct containing the indicated gene was transformed into *S. cerevisiae* along with the β-glucosidase, GH1-1. These strains were grown with cellobiose as a sole carbon source. The growth rates were determined and compared to strains with WT CDT-1 and GH1-1, or no transporter and GH1-1. Each value represents the mean of three measurements, and is reported as a percentage of the WT growth rate. Error bars represent the standard deviation between these measurements.

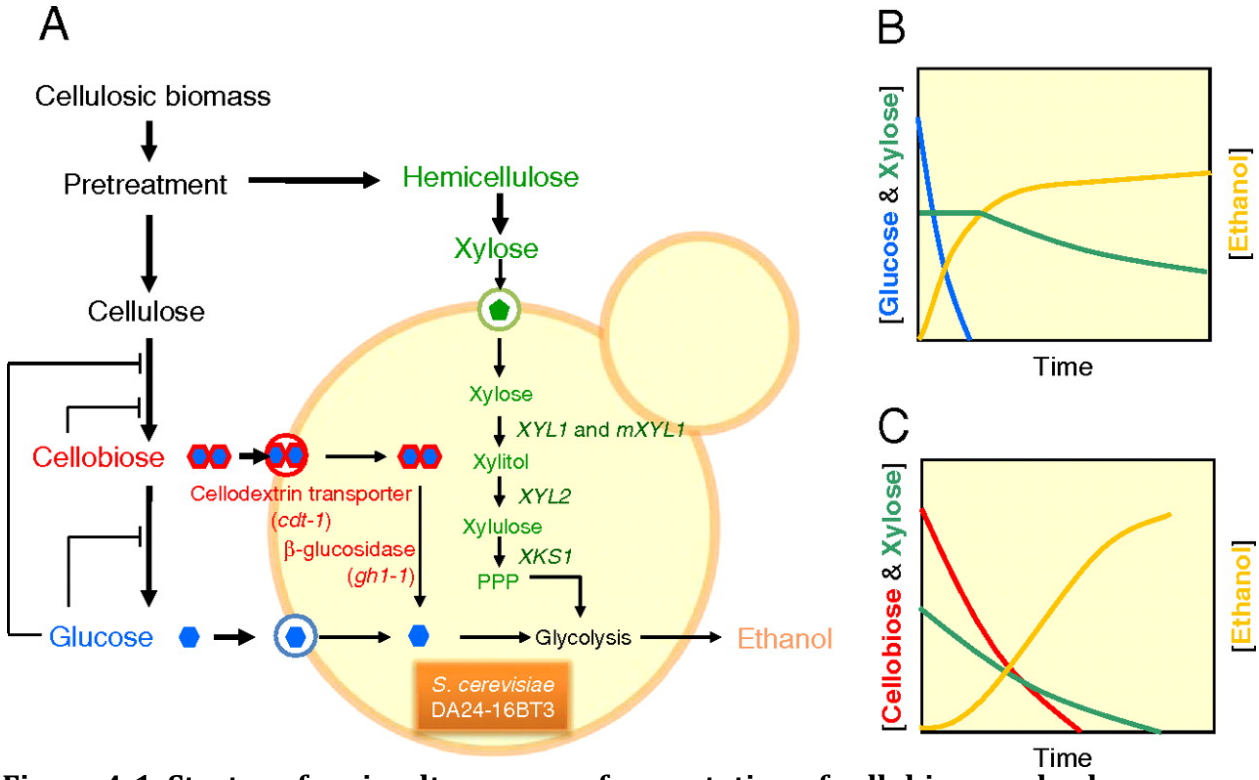

Figure 4-1. Strategy for simultaneous co-fermentation of cellobiose and xylose without glucose repression. (A) A strain improvement strategy to engineer yeast capable of fermenting two non-metabolizable sugars (cellobiose and xylose). The cellodextrin assimilation pathway consists of a cellodextrin transporter (CDT-1) and an intracellular β-glucosidase (GH1-1) from the filamentous fungus *N. crassa*. The modified xylose metabolic pathway utilizes xylose reductase isoenzymes (wild-type XR and a mutant XRR276H), xylitol dehydrogenase (XYL2), and xylulokinase (XKS1) from the xylose-fermenting yeast *P. stipitis*. **(B)** Schematic fermentation profile of a sugar mixture containing glucose and xylose by the engineered *S. cerevisiae*. Glucose fermentation represses xylose fermentation completely so that xylose fermentation begins only after glucose depletion. **(C)** Schematic fermentation profile of a sugar mixture containing cellobiose and xylose by the engineered *S. cerevisiae*. Cellobiose and xylose are simultaneously utilized, as neither carbon source represses consumption of the other.

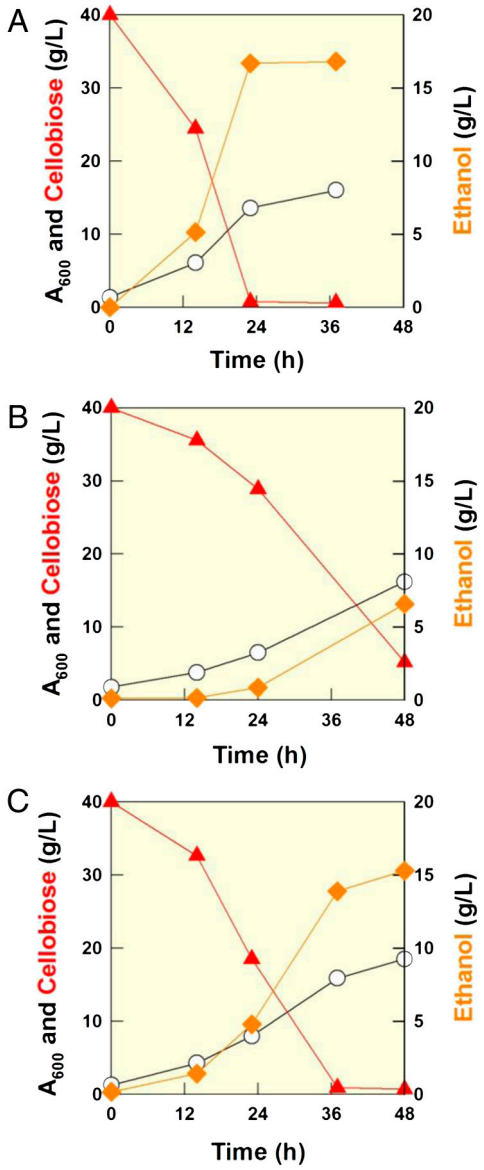

Figure 4-2. Cellobiose fermentation by *S. cerevisiae* with three cellobiose transporters. *S. cerevisiae* was transformed with the β-glucosidase, *gh1-1*, and either *cdt-1* **(A)**, NCU00809 **(B)** or *cdt-2* **(C)**. The capacity of these strains to ferment 40 g/L cellobiose to ethanol was measured. Charts show the concentration of cellobiose (red triangles) and ethanol (orange diamonds), and the amount of biomass present (white circles).

Strains	Specific XR activity (U/mg)		Ratio of XR activity with NADH & NADPH	Reference (citation details in legend)
	NADH	NADPH		
S. cerevisiae with wild-type XYL1	0.07 ± 0.03	0.09 ± 0.02	0.8	Amore et al. (1)
"	0.10 ± 0.02	0.12 ± 0.03	0.8	Takuma et al. (2)
"	-	2.7 ± 2.5	-	Walfridsson et al. (3)
"	2.3 ± 0.3	3.4 ± 0.5	0.68	Tantirungkij et al. (4)
"	-	0.411	-	Ho et al. (5)
"	-	0.43 ± 0.03	-	Eliasson et al. (6)
"	-	1.1 ± 0.08	-	Wahlbom et al. (7)
S. cerevisiae with mutant XYL1	9.3 ± 0.1	0.4 ± 0.0	23.1	Watanabe et al. (8)
DA24	0.32 ± 0.15	0.29 ± 0.10	1.1	This study

Table 4-3. Comparison of xylose reductase activity of DA24 with those of other engineered *S. cerevisiae* strains expressing wild-type or mutant *XYL1*. References: (1), (Amore, Kotter, Kuster, Ciriacy, & Hollenberg, 1991); (2), (Takuma et al., 1991); (3), (Walfridsson, Anderlund, Bao, & Hahn-Hagerdal, 1997); (4), (Tantirungkij, Seki, & Yoshida, 1994); (5), (Ho, et al., 1998); (6), (Eliasson, Christensson, Wahlbom, & Hahn-Hagerdal, 2000); (7), (Wahlbom, van Zyl, Jonsson, Hahn-Hagerdal, & Otero, 2003); (8), (Watanabe, et al., 2007).

Carbon source	Strain	Ethanol produced (g/L)	Sugar consumption rate (g/L-h)	Yield (g/g)	Productivity (g/L-h)
Xylose (80 g/L)	DA24	24.2	1.16	0.34	0.40
	DA24-16	27.9	1.32	0.35	0.47
Glucose (80 g/L) & Xylose (40 g/L)	DA24	34.8	1.45	0.39	0.74
	DA24-16	45.1	1.78	0.42	0.96

Table 4-4. Comparison of fermentation parameters by DA24 and DA24-16 under two different sugar conditions.

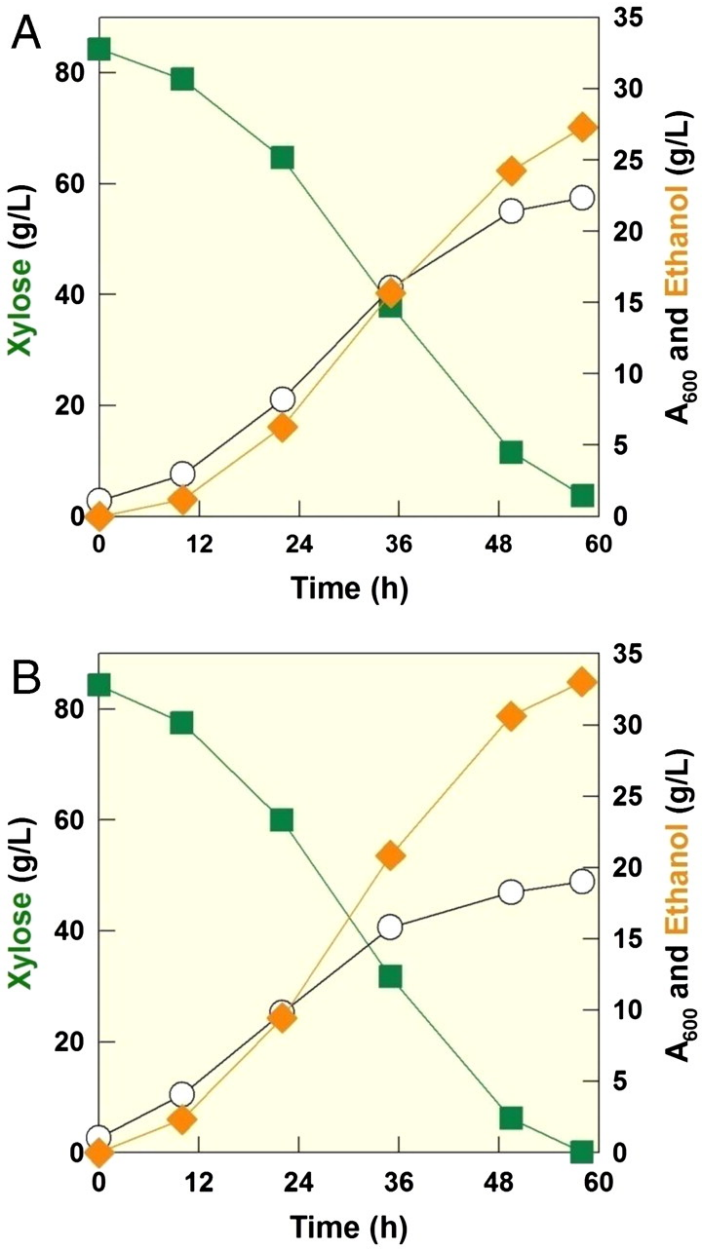

Figure 4-5. Comparisons of xylose consumption and ethanol production between DA24-16 (A) and *Pichia stipitis* (B). Symbols: xylose (green square), ethanol (orange diamond), and cell growth (open circle).

Cellobiose/Xylose (g/L)	Ethanol produced (g/L)	Sugar consumption rate (g/L-h)	Yield (g/g)	Productivity (g/L-h)
20/20	14.8	1.12	0.37	0.41
30/30	23.3	1.33	0.37	0.50
40/40	30.5	1.67	0.39	0.65

Table 4-6. Co-fermentation of cellobiose and xylose using different mixtures of the two sugars.

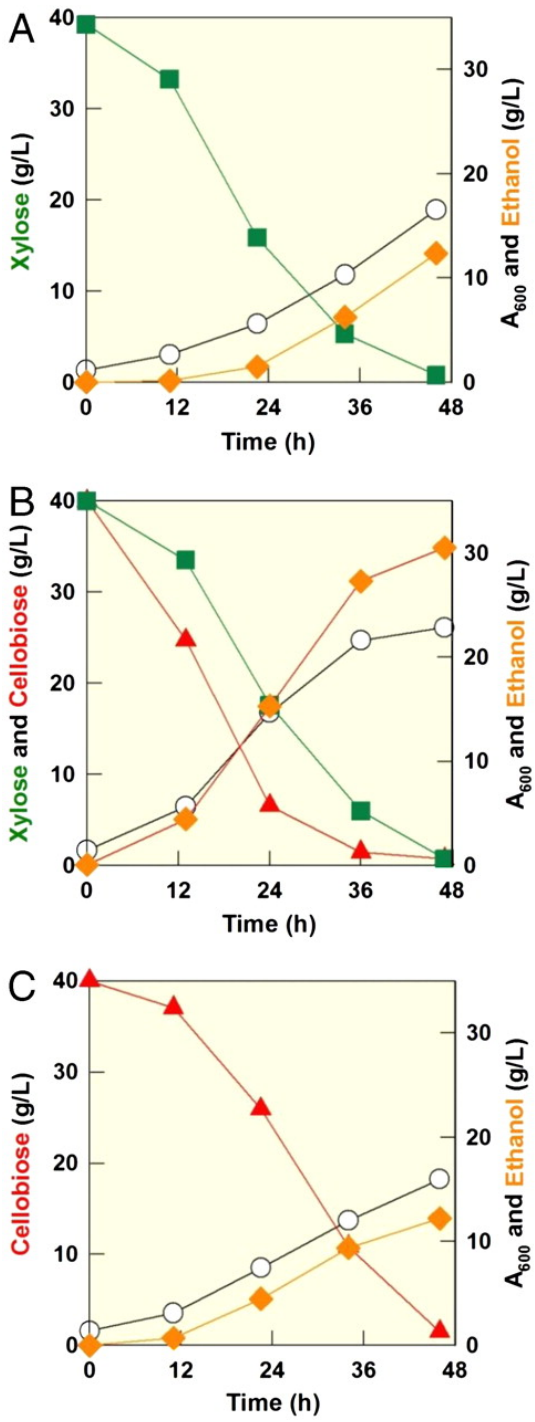

Figure 4-7. Synergistic effects from co-fermentation of cellobiose and xylose by strain DA24-16BT3. Symbols: cellobiose (red triangle up), xylose (green square), ethanol (orange diamond), and cell growth (open circle). **(A)** 40 g/L of cellobiose, **(B)** a mixture of 40 g/L of cellobiose and 40 g/L of xylose, and **(C)** 40 g/L of xylose.

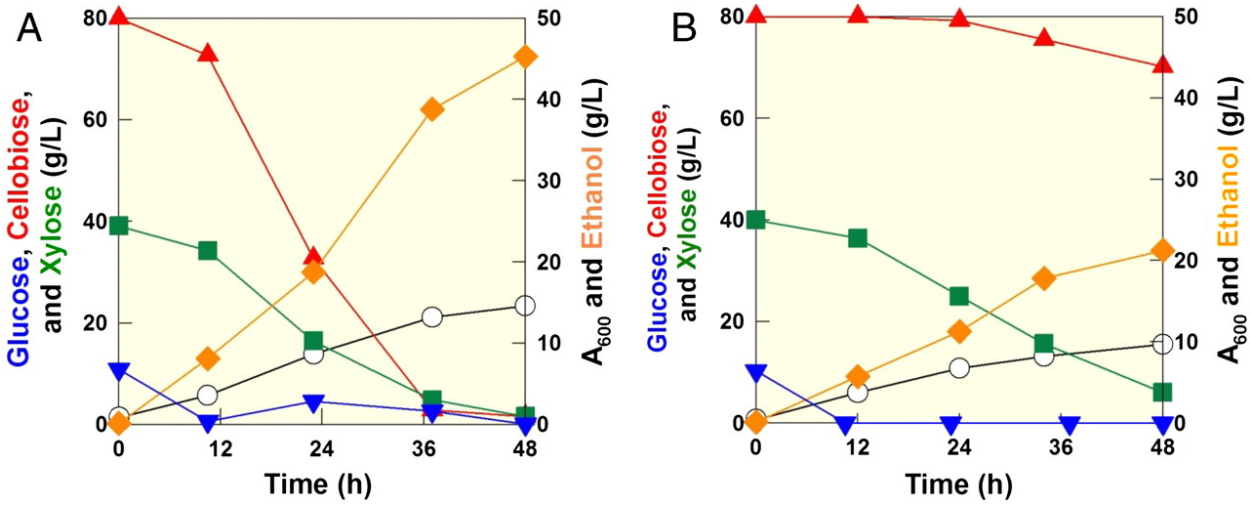

Figure 4-8. Co-fermentation of glucose, cellobiose, and xylose by strain DA24-16BT3 and *Pichia stipitis*. (A) DA24-16BT3 fermentation profile of a sugar mixture containing 80 g/L of cellobiose, 40 g/L of xylose, and 10 g/L of glucose. **(B)** *P. stipitis* fermentation of a sugar mixture containing 80 g/L of cellobiose, 40 g/L of xylose, and 10 g/L of glucose. Symbols: cellobiose (red triangle up), xylose (green square), ethanol (orange diamond), OD (open circle), and glucose (blue triangle down).

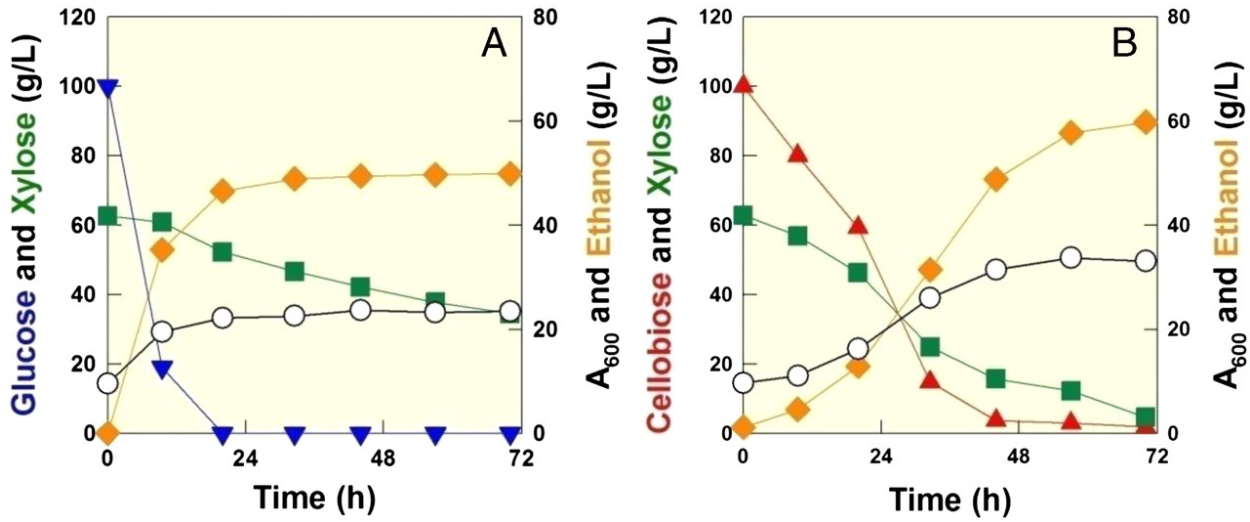

Figure 4-9. Fermentation profiles by DA24-16BT3 during the fermentation of sugar mixtures containing extreme concentrations of glucose and xylose. (A) 100 g/L of glucose and 60 g/L of xylose and cellobiose and xylose, **(B)** 100 g/L of cellobiose and 60 g/L xylose. Actual fermentation profiles are similar to the patterns of sugar utilization and ethanol production illustrated in Fig. 3-1. Symbols: cellobiose (red triangle up), xylose (green square), ethanol (orange diamond), OD (open circle), and glucose (blue triangle down).

Strain	Description	Reference (detail in legend)
D452-2	*MATa, leu2, his3, ura3, can1*	Hosaka et al. (1)
D801-130	D452-2 expressing *gh1-1* and *cdt-1*	This study
D809-130	D452-2 expressing *gh1-1* and NCU00809	This study
D8114-130	D452-2 expressing *gh1-1* and *cdt-2*	This study
DA24	D452-2 expressing *XYL1, mXYL1, XYL2, and XKS1* (Isogenic of D452-2 except for *leu2 ::* $TDH3_P$*-XYL1-*$TDH3_T$*, ura3 ::URA3-*PGK_P*-mXYL1-*PGK_T*-*PGK_P*-XYL2-*PGK_T*, Ty3 ::neo-*TDH_P*-XKS1-*TDH_T)	This study
DA24-16	Evolved strain of DA24 in xylose containing media	This study
DA24-16BT3	DA24-16 expressing *gh1-1* in a multicopy plasmid and *cdt-1* through single-copy integration	This study
DA24-16BT-M	DA24-16 expressing *gh1-1* and *cdt-1* in multicopy plasmids	This study
P. stipitis CBS 6054	NRRL Y-11545 = ATCC58785 = IFO10063	Jeffries et al. (2)
pRS425	*LEU2*, a multicopy plasmid	Christianson et al. (3)
pRS426	*URA3*, a multicopy plasmid	Christianson et al. (3)
pRS403	*HIS3*, an integrative plasmid	Sikorski et al. (4)
pRS405	*URA3*, an integrative plasmid	Sikorski et al. (4)
pRS425-*gh1-1*	*gh1-1* under the control of PGK promoter in pRS425	Galazka et al. (5)
pRS426-*cdt-1*	*cdt-1* under the control of PGK promoter in pRS426	Galazka et al. (5)
pRS426-*cdt-2*	*cdt-2* under the control of PGK promoter in pRS426	Galazka et al. (5)
pRS426-NCU00809	NCU00809 under the control of PGK promoter in pRS426	Galazka et al. (5)
pRS403-*cdt-1*	*cdt-1* under the control of PGK promoter in pRS403	This study

Table 4-10. Strains and plasmids used in this study. References: (1) (Hosaka, et al., 1992); (2) (T. W. Jeffries, et al., 2007); (3) (Christianson, Sikorski, Dante, Shero, & Hieter, 1992); (4) (Sikorski & Hieter, 1989); (5) (Galazka, et al., 2010).

Name	Sequence
NCU00801-F	ATGGATCCAAAAATGTCGTCTCACGGCTCC
NCU00801-R	ATGAATTCCTACAAATCTTCTTCAGAAATCAATTTTTGTTCAGCAACGATAGCTTCGGAC
NCU08114-F	ATACTAGTAAAAATGGGCATCTTCAACAAGAAGC
NCU08114-R	GCATATCGATCTACAAATCTTCTTCAGAAATCAATTTTTGTTCAGCAACAGACTTGCCCTCATG
NCU00130-F	GCATACTAGTAAAAATGTCTCTTCCTAAGGATTTCCTCT
NCU00130-R	ATACTGCAGTTAATGATGATGATGATGATGGTCCTTCTTGATCAAAGAGTCAAAG

Table 4-11. Synthetic oligonucleotides used in this study.

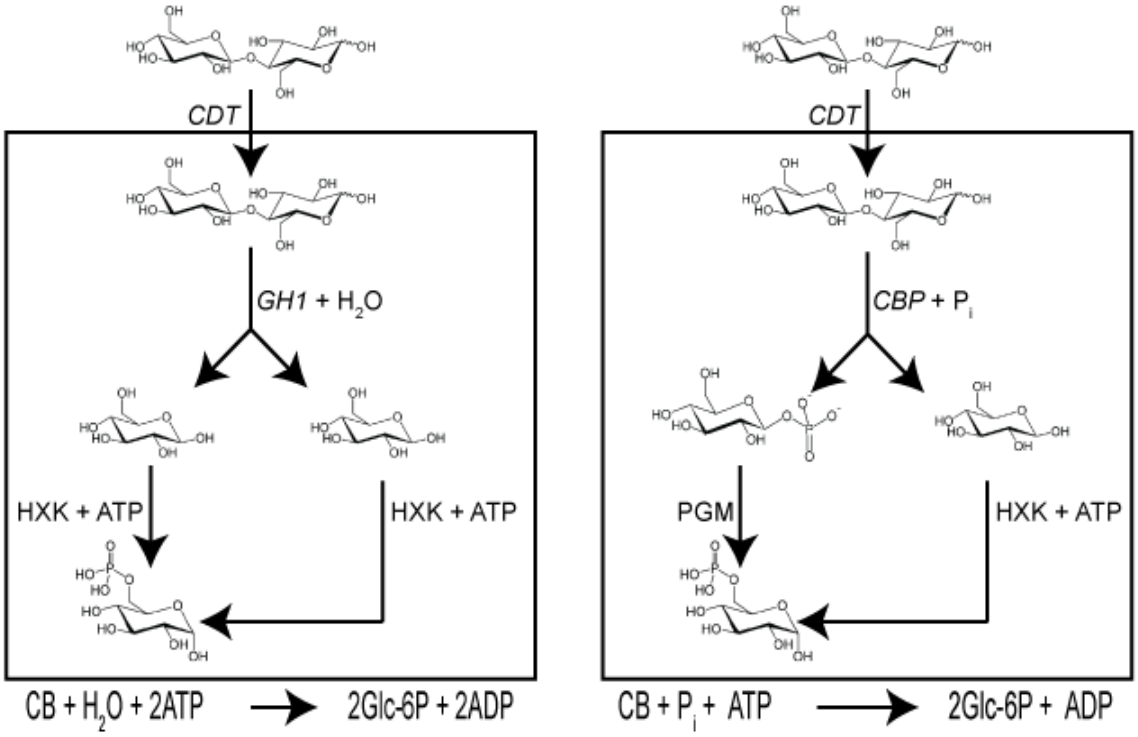

Figure 5-1. Two possible cellobiose fermentation pathways. Following transport across the plasma membrane by a cellodextrin transporter (CDT), cellobiose is cleaved either by hydrolysis (left panel) *via* β-glucosidase (GH1) or phosphorolysis (right panel) *via* cellobiose phosphorylase (CBP). Intracellular glucose is formed in the hydrolytic pathway, and this is converted to glucose-6-phosphate (G6P) by hexokinase (HXK). Intracellular glucose and glucose-1-phosphate is formed in (G1P) the phosphorolytic pathway. Here, G1P is converted to G6P by phosphoglucomutase (PGM), while glucose is converted to G6P by HXK. In both pathways G6P is fermented to ethanol and CO_2 by endogenous yeast enzymes. Enzymes engineered into yeast are italicized.

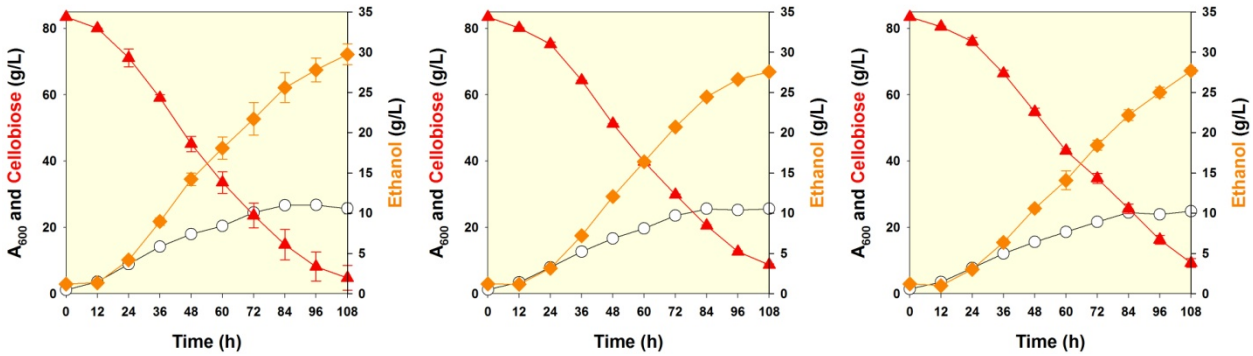

Figure 5-2. Cellobiose fermentation profile of three yeast strains each with CDT-1 and one of three cellobiose phosphorylase genes. The D452-2 strain of *S. cerevisiae* was transformed with one plasmid containing *cdt-1*, and a second plasmid containing a codon-

optimized cellobiose phosphorylase from *C. gilvus* (left), *S. degradans* (middle), or *C. thermocellum* (right). The rate at which these strains fermented cellobiose (red triangles) to ethanol (orange diamonds) was determined under oxygen limited conditions. The amount of produced biomass was also monitored (white circles). Each profile was run independently twice, and there was less than 5% variance between replicates. Shown is a representative profile.

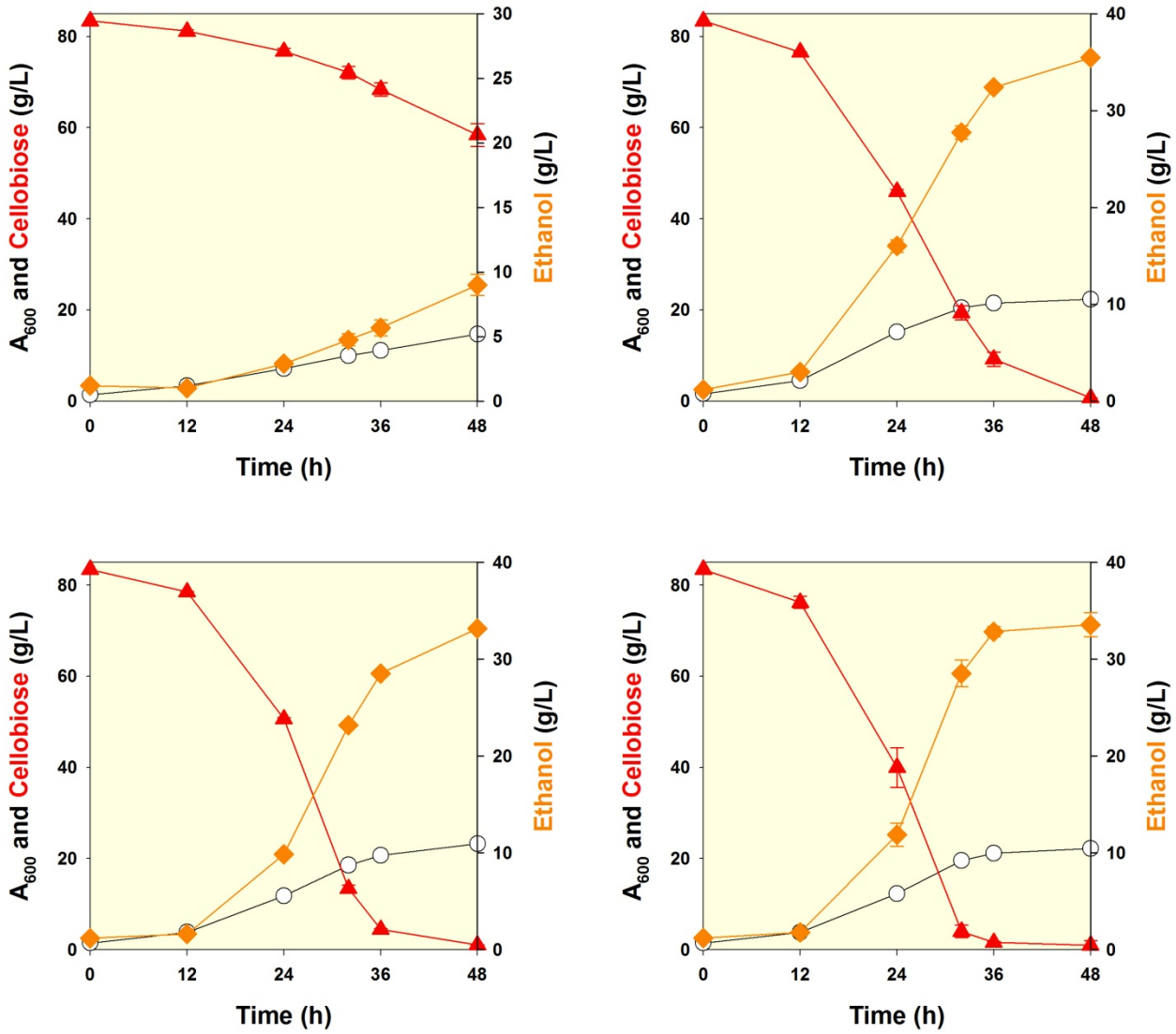

Figure 5-3. Fermentation profile of strains bearing a spontaneously derived mutant of CDT-1. (A) *S. cerevisiae* was transformed with one plasmid containing either the WT cellobiose transporter, CDT-1, (left panel) or an evolved mutant of this transporter, CDT-1(F213L), (right panel) and a second plasmid with the cellobiose phosphorylase, SdCBP. The rate at which these strains fermented cellobiose (red triangles) to ethanol (orange diamonds) was determined under oxygen limited conditions. The amount of produced biomass was also monitored (white circles). **(B)** *S. cerevisiae* was transformed with one plasmid containing either the WT cellobiose transporter, CDT-1, (left panel) or an evolved

mutant of this transporter, CDT-1(F213L), (right panel) and a second plasmid with the cellobiose hydrolase, 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.

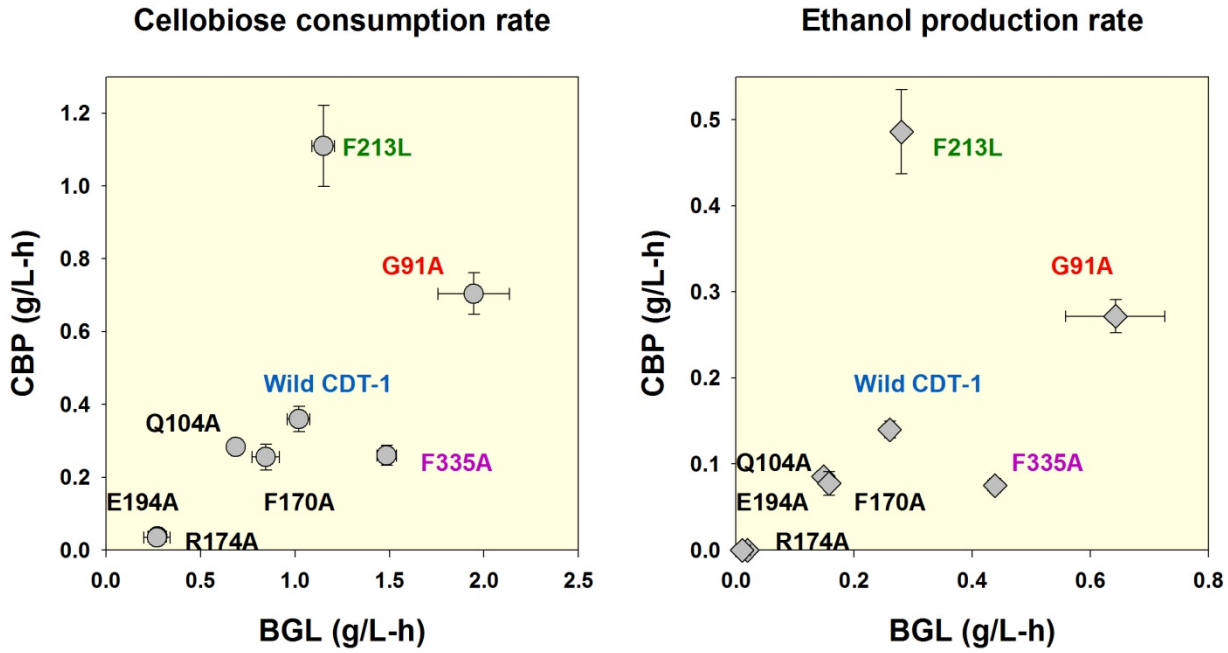

Figure 5-4. Cellobiose consumption and ethanol production of strains with various CDT-1 mutants. The D452-2 strain of *S. cerevisiae* was transformed with one plasmid containing either WT *cdt-1* or a *cdt-1* mutant, and a second plasmid containing codon-optimized cellobiose phosphorylase from *S. degradans* or the β-glucosidase, *gh1-1*. The rate at which these strains consumed cellobiose (left panel) and produced ethanol (right panel) is shown. Productivities with various *cdt-1* mutants and cellobiose phosphorylases are along the y-axis, while productivities with various *cdt-1* mutants and β-glucosidase are along the x-axis. All values are the mean of two independent fermentations, and error bars represent the standard deviations between these.

Cellobiose consumption rate (g/L-h)			Ethanol production rate (g/L-h)		
	With BGL	With CBP		With BGL	With CBP
Wild CDT-1	1.02±0.0600	0.36±0.0350	Wild CDT-1	0.26±0.0100	0.2718±0.0100
G91A	1.95±0.1899	0.70±0.0566	G91A	0.64±0.0842	0.0855±0.0193
Q104A	0.69±0.0373	0.28±0.0037	Q104A	0.15±0.0100	0.0775±0.0022
F170A	0.85±0.0730	0.26±0.0351	F170A	0.16±0.0104	0.0000±0.0136
R174A	0.27±0.0475	0.04±0.0028	R174A	0.02±0.0020	0.0000±0.0000
E194A	0.27±0.0693	0.03±0.0050	E194A	0.01±0.0144	0.0750±0.0000
F335A	1.49±0.0520	0.26±0.0276	F335A	0.44±0.0113	0.1400±0.0081
F213L	1.15±0.0600	1.11±0.1110	F213L	0.28±0.0100	0.4860±0.0490

Table 5-5. Cellobiose consumption and ethanol production of strains with various CDT-1 mutants. Values correspond to those in figure 5-4.

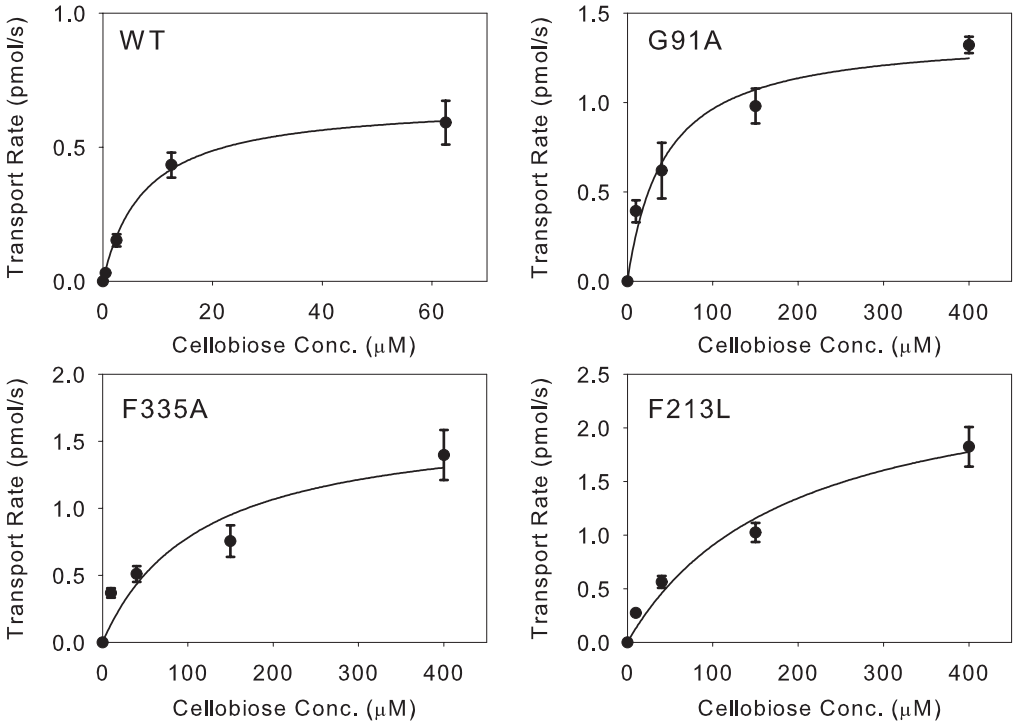

Figure 5-6. Transport kinetics of WT CDT-1 and 3 CDT-1 mutants. The linear rate of [^{3}H]-cellobiose uptake into yeast strains with CDT-1, CDT-1(F213L), CDT-1(F335A), or CDT-1(G91A) was determined using various concentrations of cellobiose. Error bars represent the standard deviation of three replicate measurements at each concentration.

	K_M (μM)	V_{max} (pmol/s, norm. to $2x10^8$ cells)	V_{max} (pmol/s, norm. for GFP fluo.)
WT	7.6 ± 1.5	0.60 ± 0.03	0.86 ± 0.05
F213L	188.8 ± 55.3	2.66 ± 0.37	2.38 ± 0.31
F335A	114.8 ± 46.4	1.64 ± 0.24	1.75 ± 0.26
G91A	43.5 ± 10.1	1.51 ± 0.10	1.87 ± 0.12

Table 5-7. Transport kinetics of WT CDT-1 and 3 CDT-1 mutants. Kinetic parameters reported in the table were determined *via* non-linear regression from the datasets in figure 5-6.

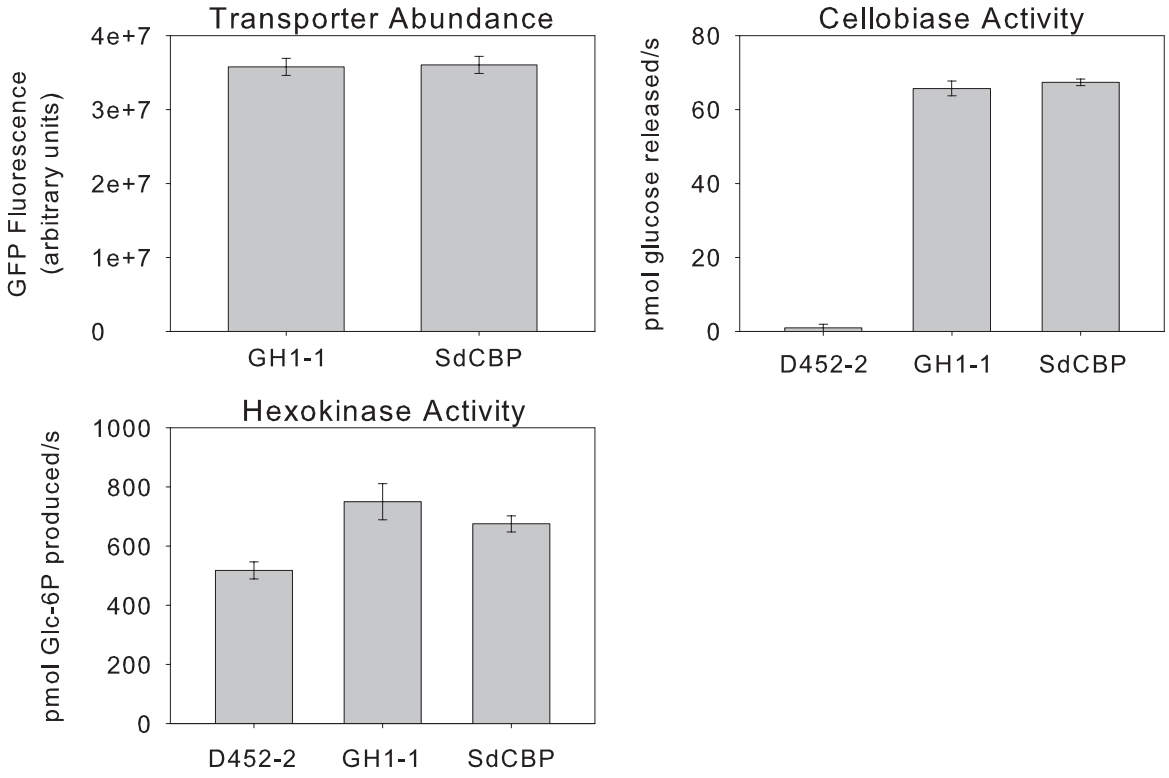

Figure 5-7. Activity of GH1-1, *S. degradans* cellobiose phosphorylase and *S. cerevisiae* hexokinases in cell extracts. Cell extracts were harvested from D452-2 growing on rich glucose media (YPD80), and from D452-2 expressing a cellobiose catabolism pathway consisting of CDT-1 and either GH1-1 or the *S. degradans* cellobiose phosphorylase growing on rich cellobiose media (YPC80). The amount of hexokinase activity, or cellobiase activity (defined as the rate of glucose released regardless of the mechanism) in 10 μg of extract was determined. In addition the amount of transporter present in each strain was estimated by measuring GFP fluorescence. Results are the mean ± the standard deviation of three cultures.

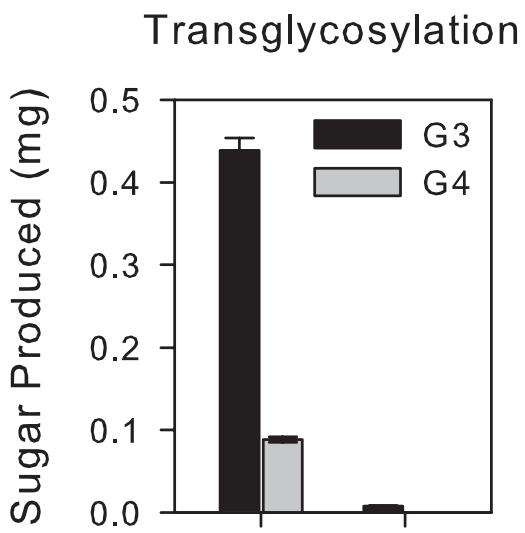

Figure 5-8. Transglycosylation activity of GH1-1 and *S. degradans* cellobiose phosphorylase. 200 pkat of either purified GH1-1 or *S. degradans* cellobiose phosphorylase was incubated with 20% (w/v) cellobiose for 24 hours @ 30 °C in 50 mM phosphate buffer at pH 6.0. The same incubation was carried out without the addition of enzyme as a control. The products were then analyzed by HPLC.

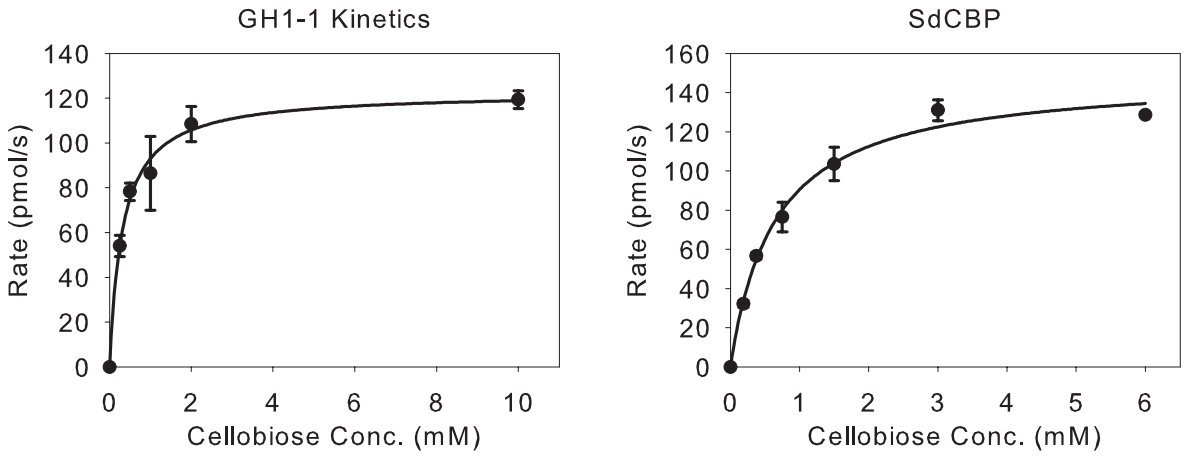

Figure 5-9. Michaelis Menten Analysis of purified GH1-1 and SdCBP. GH1-1 and the *S. degradans* cellobiose phosphorylase were purified directly from the strains being studied. Linear rates of catalysis were determined at a variety of cellobiose concentrations in triplicate, and kinetic parameters determined by non-linear regression.

	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M
GH1-1	0.32	7	21910
SdCBP	0.65	7	11515

Table 5-10. Kinetic parameters of GH1-1 and SdCBP. Kinetic parameters reported in the table were determined *via* non-linear regression from the datasets in **figure 4-9**.

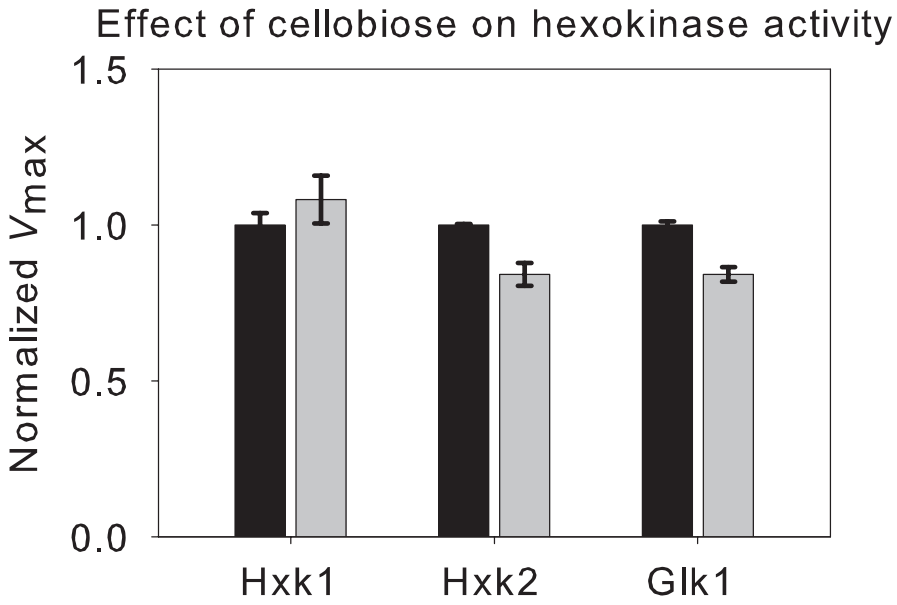

Figure 5-11. Effect of cellobiose on activity of purified hexokinase. The three *S. cerevisiae* hexokinases were expressed and purified from *E. coli*. To determine the effect of cellobiose on hexokinase activity, linear rates of glucose-phosphorylating activity were determined in the presence (grey bars) or absence (black bars) of 184 mM cellobiose. Results are the mean ± the standard deviation of triplicate measurements.

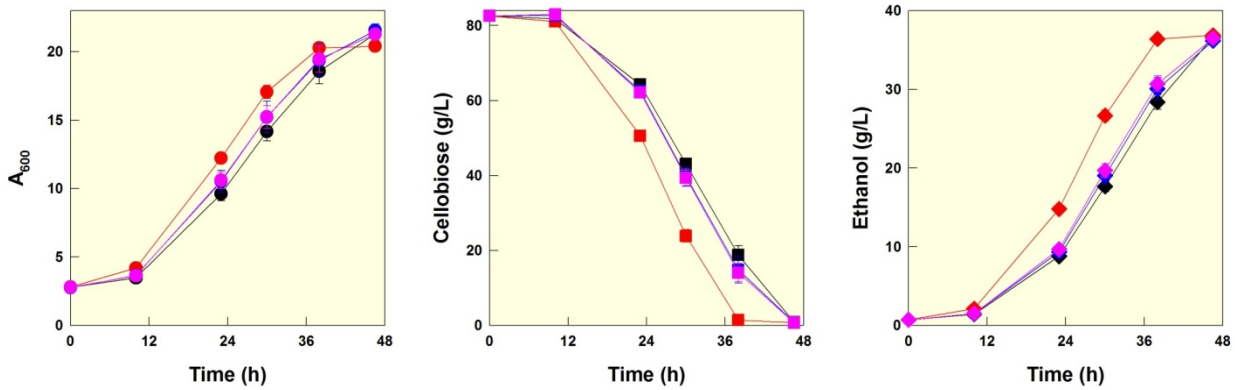

Figure 5-12. Over-expression of Hxk1 improves the phosphorolytic pathway. Two hexokinases (*HXK1*, *HXK2*), and one glucokinase (*GLK1*) were over-expressed in *S. cerevisiae* along with CDT-1(F213L) and the phosphorylase from *S. degradans*. The capacity of strains with *HXK1* (red symbols), *HXK2* (blue symbols), *GLK1* (violet symbols) or an empty plasmid (black symbols) to ferment 80 g/L cellobiose to ethanol was monitored. The panels show cell growth (left), cellobiose concentration (middle) and ethanol concentration (right).

	OD (600 nm)	Ethanol (g/L)	Yield (g/g)	Productivity (g/L-h)
pRS423	19	28	0.46	0.75
pRS423-Hxk1	20	36	0.46	0.96 (28% ↑)
pRS423-Hxk2	19	30	0.46	0.79
pRS423-Glk1	19	31	0.46	0.81

Table 5-13. Cellobiose fermentation parameters of *S. cerevisiae* over-expressing *HXK1*, *HXK2* or *GLK1* along with a phosphorolytic cellobiose consumption pathway. Two hexokinases (*HXK1*, *HXK2*), and one glucokinase (*GLK1*) were over-expressed separately in *S. cerevisiae* along with CDT-1(F213L) and the phosphorylase from *S. degradans*. The capacity of these strains to ferment 80 g/L cellobiose to ethanol was measured. Fermentation parameters were determined from the charts in **figure 5-12**.

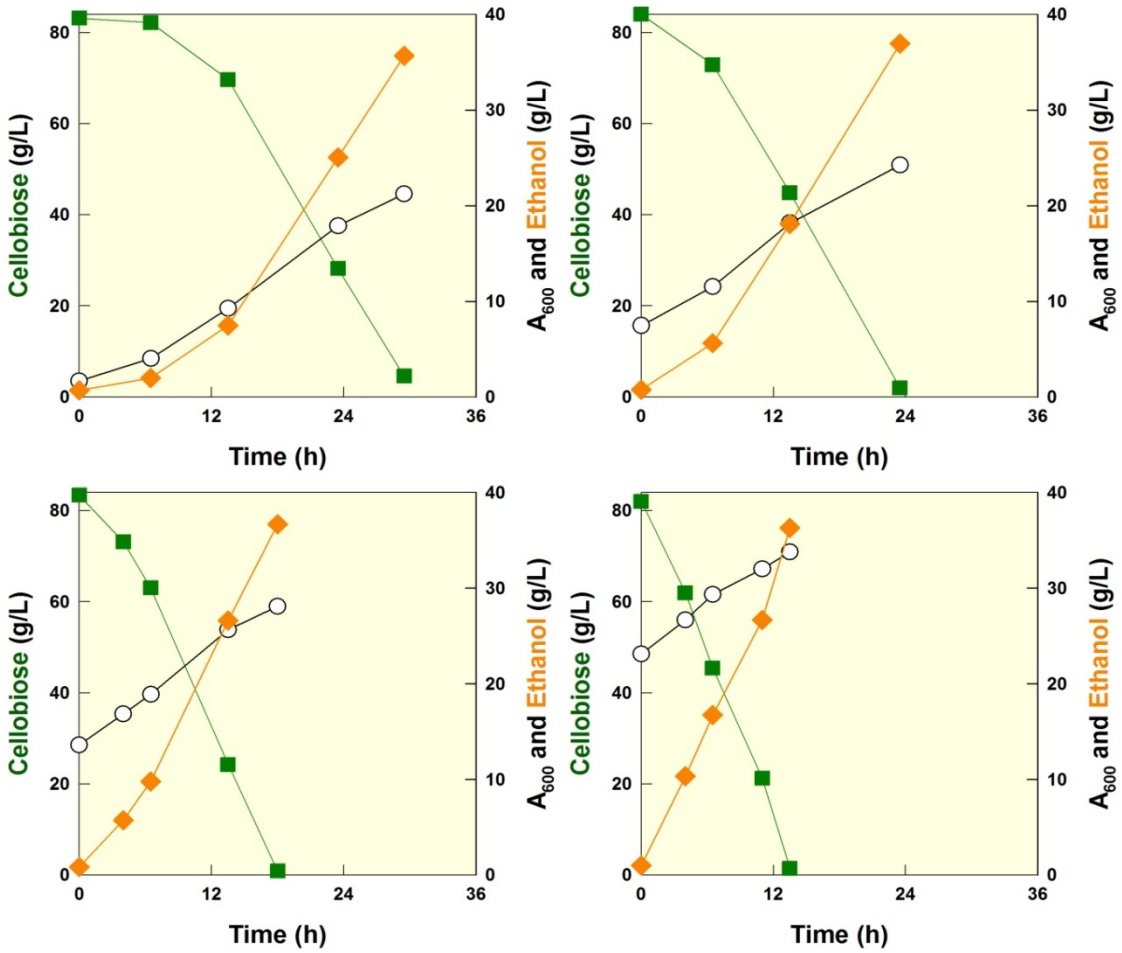

Figure 5-14. Productivity of strains over-expressing *HXK1* and an efficient phosphorolytic pathway increases with higher cell inoculums. The cellobiose fermentation capacity of *S. cerevisiae* strains over-expressing *HXK1* along with CDT-1(F213L) and the *S. degradans* CBP was measured with different cell inoculums. Cell inoculums of OD (A600) 1.6 (top left), 7.5 (top right), 13.6 (bottom left) and 23.1 (bottom right) were tested. In all panels the concentration of cellobiose (green squares) and ethanol (orange diamonds) is shown along with the amount of biomass (white circles).

Initial OD	1.6	7.5	13.6	23.1
OD (A600)	21	24	28	34
Ethanol (g/L)	36	37	37	36
Y (g/g)	0.47	0.47	0.46	0.46
P (g/L-h)	1.21	1.57	2.04	2.69

Table 5-15. Fermentation parameters of strains over-expressing *HXK1* and an efficient phosphorolytic pathway at different inoculums. The cellobiose fermentation capacity of *S. cerevisiae* strains over-expressing *HXK1* along with CDT-1(F213L) and the *S. degradans* CBP was measured with different cell inoculums. Cell inoculums 1.6, 7.5, 13.6 and 23.1 were tested. Parameters were determined from the charts in **figure 5-14**.

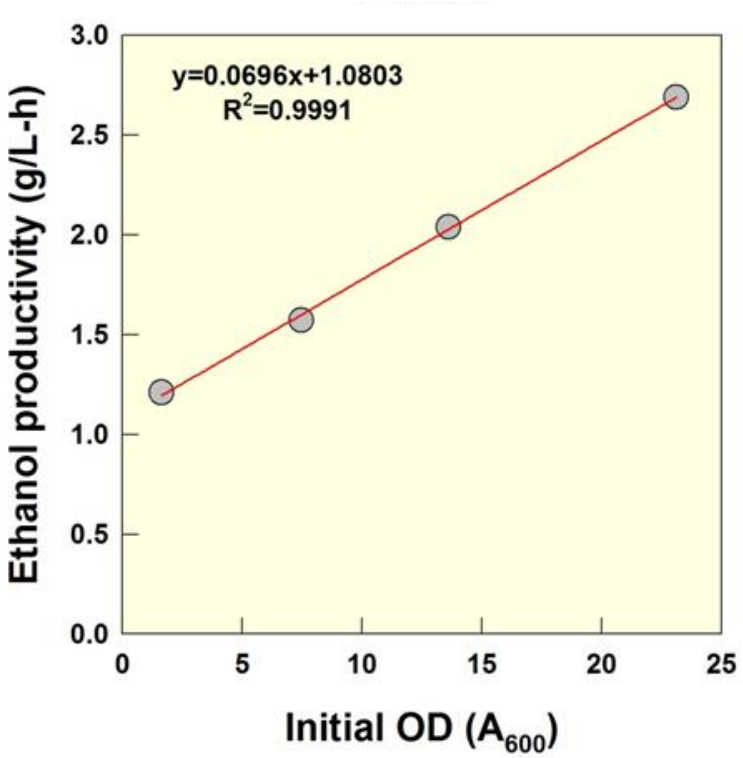

Figure 5-16. Productivity of strains over-expressing *HXK1* and an efficient phosphorolytic pathway increases linearly with cell inoculum. The cellobiose fermentation capacity of *S. cerevisiae* strains over-expressing *HXK1* along with CDT-1(F213L) and the *S. degradans* CBP was measured with different cell inoculums. The cellobiose fermentation capacity of *S. cerevisiae* strains over-expressing *HXK1* along with CDT-1(F213L) and the *S. degradans* CBP was measured with different cell inoculums. Cell inoculums 1.6, 7.5, 13.6 and 23.1 were tested. Productivities are taken from the **table 5-15**.

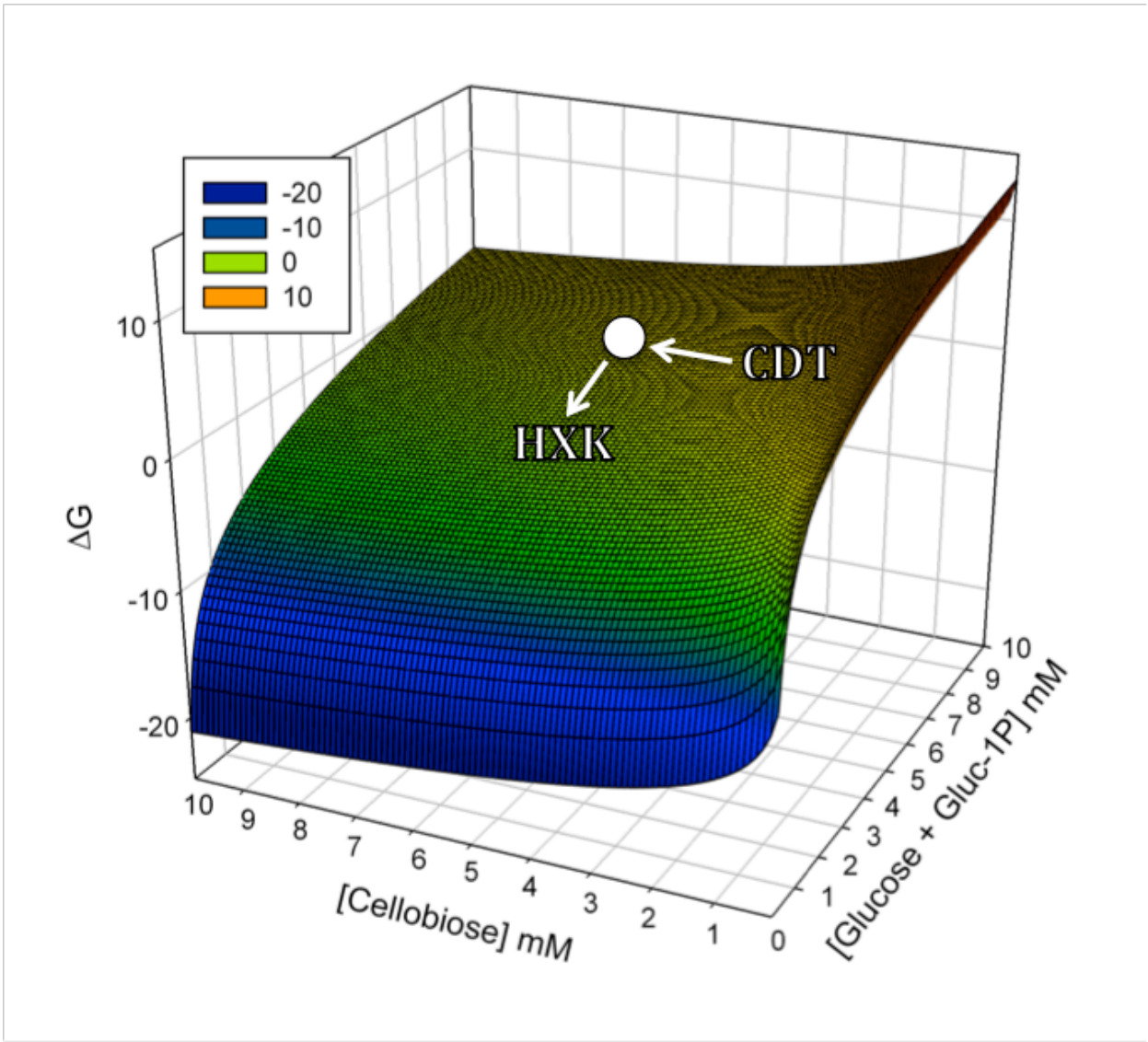

Figure 5-17. Cellobiose phosphorolysis must be "pushed" by transporter mutants and "pulled" by hexokinase over-expression. Shown is a plot of the calculated ΔG of the cellobiose phosphorolysis reaction using the following equation: $\Delta G = \Delta G^\circ + RT \ln [glucose][Gluc\text{-}1P]/[cellobiose][P_i]$ where ΔG° = +3.4 kJ/mol-K, T = 298.15 K and $[P_i]$ = 22 mM. The ΔG was modeled for cellobiose and glucose + Gluc-1P concentrations of 0 – 10 mM. The circle indicates the ΔG at an arbitrary condition and arrows indicate the affect that an increase in the rate of cellobiose transport (CDT), or glucose phosphorylation (HXK), would have on this ΔG.

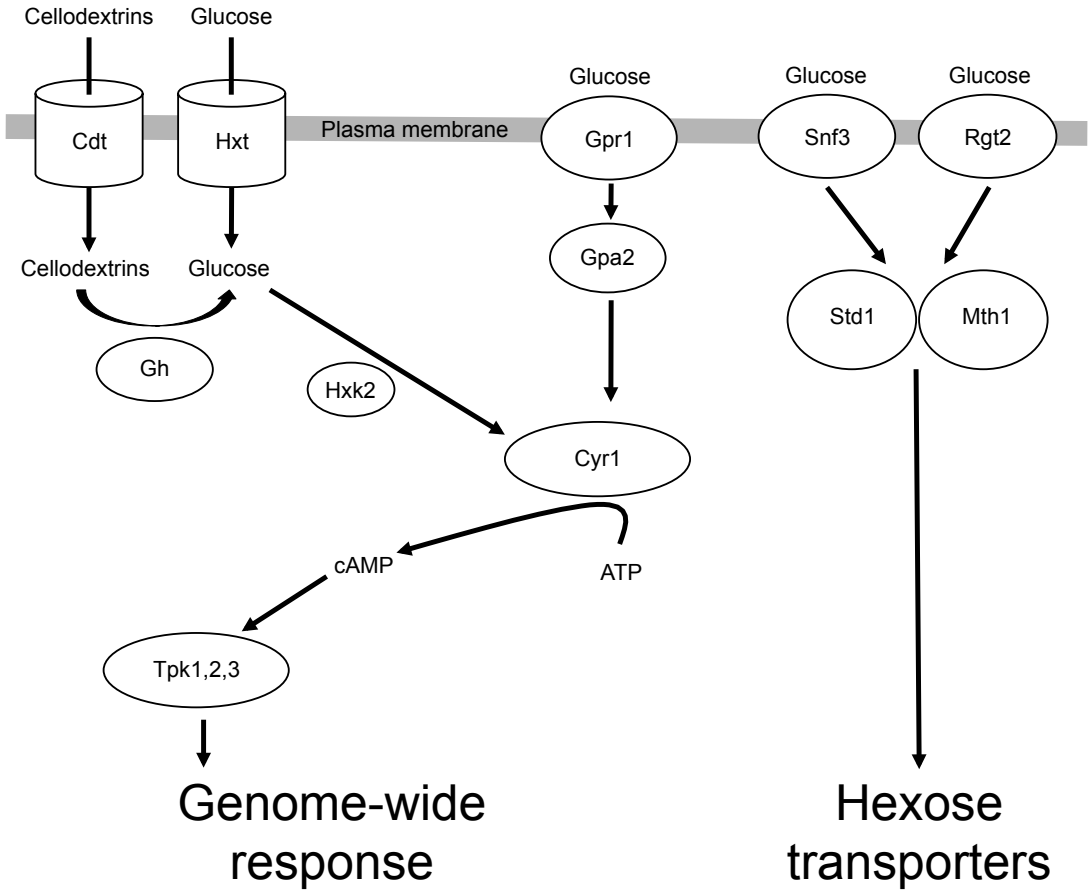

Figure 6-1. **Model of the glucose sensing network of *S. cerevisiae* in the context of a cellodextrin transport pathway.** Glucose is sensed by both extracellular and intracellular systems. Extracellular glucose is sensed by the 7-transmembrane protein, Gpr1, as well as the non-transporting transceptors, Snf3 and Rgt2. Intracellular glucose, which can enter through the hexose transport system (Hxt), is sensed by a pathway that involves Hxk2. Signals emanating from Gpr1 and intracellular glucose converge to induce a genome-wide transcriptional response through the Ras/PKA pathway, which includes adenylate cyclase (Cyr1) and protein kinase A (Tpk1,2,3). Signals emanating from Snf3 and Rgt2 modulate the expression of various hexose transporters through Std1 and Mth1. A cellodextrin transport pathway would likely bypass Gpr1, Snf3, or Rgt2, but may activate the Ras/PKA pathway following transport and hydrolysis of cellodextrins. This figure is a simplified model adapted from Rolland et al. (Rolland, Winderickx, & Thevelein, 2002).