Directed Evolution of a Cellodextrin Transporter for Improved Biofuel Production Under Anaerobic Conditions in *Saccharomyces cerevisiae*

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**ABSTRACT:** Introduction of a cellobiose utilization pathway consisting of a cellodextrin transporter and a β-glucosidase into *Saccharomyces cerevisiae* enables co-fermentation of cellobiose and xylose. Cellodextrin transporter 1 (CDT1) from *Neurospora crassa* has been established as an effective transporter for the engineered cellobiose utilization pathways. However, cellodextrin transporter 2 (CDT2) from the same species is a facilitator and has the potential to be more efficient than CDT1 under anaerobic conditions due to its energetic benefits. Currently, CDT2 has a very low activity and is considered rate-limiting in cellobiose fermentation. Here, we report the directed evolution of CDT2 with an increased cellobiose uptake activity, which results in improved cellobiose fermentation under anaerobic conditions. After three rounds of directed evolution, the cellobiose uptake activity of CDT2 was increased by 2.2-fold, which resulted from both increased specific activity and transporter expression level. Using high cell density fermentation under anaerobic conditions, the evolved mutant conferred 4.0- and 4.4-fold increase in the cellobiose consumption rate and ethanol productivity, respectively. In addition, although the cellobiose uptake activity was still lower than that of CDT1, the engineered CDT2 showed significantly improved cellobiose consumption and ethanol production under anaerobic conditions, representing the energetic benefits of a sugar facilitator for anaerobic cellobiose fermentation. This study demonstrated that anaerobic biofuel production could be significantly improved via directed evolution of a sugar transporter protein in yeast.


**KEYWORDS:** cellodextrin transporter; cellobiose utilization; cellulosic biofuel; anaerobic fermentation; directed evolution

**Introduction**

Biological conversion of plant-derived lignocellulosic materials into biofuels has been intensively investigated as a result of increasing concerns on energy security, sustainability and global climate change (Du et al., 2011; Jang et al., 2012). *Saccharomyces cerevisiae*, also known as baker’s yeast, which has been used for alcohol fermentation for thousands of years, is an excellent organism for cellulosic biofuel production (Hong and Nielsen, 2012; Sun et al., 2012). Unfortunately, wild type *S. cerevisiae* strains cannot utilize pentose sugars, especially xylose, the second most abundant carbohydrate component in lignocellulosic biomass. Heterologous xylose utilization pathways, including the bacterial isomerase pathway and the fungal oxo-reductive pathway, have been introduced to enable xylose metabolism (Hahn-Hagerdal et al., 2007). Although steady progress has been achieved to construct efficient xylose-fermenting yeasts after intensive engineering work, the uptake and metabolism of xylose is still completely inhibited by glucose (Ha et al., 2011a; Kim et al., 2012; Li et al., 2010). One explanation for glucose repression (or catabolite repression) lies in the lack of xylose specific transporters in *S. cerevisiae*. Thus, xylose can only be transported via the hexose transporters, whose affinity for xylose is two orders of magnitude lower than that for glucose (Du et al., 2010; Jojima et al., 2010). This glucose repression phenomenon results in sequential utilization of glucose and xylose, thus low yield and productivity of biofuels (Kim et al., 2012). Therefore, the construction of a yeast strain that can consume sugar mixtures efficiently and simultaneously is
one of the biggest challenges for cost-effective production of cellulosic biofuels.

Recently, cellodextrin transporters were identified and characterized, and co-expression of a cellodextrin transporter and an intra-cellular β-glucosidase was proposed to be an efficient strategy to eliminate glucose repression in *S. cerevisiae* (Galazka et al., 2010). In this system, cellodextrin is transported by the cellobextrin transporter and hydrolyzed by β-glucosidase to release glucose intra-cellularly. As a result, hexose transporters are used to uptake xylose exclusively. Using this strategy, glucose repression was eliminated and co-fermentation of cellodextrin/xylose (Fox et al., 2012; Ha et al., 2011a; Li et al., 2010) and cellodextrin/galactose (Ha et al., 2011b) were achieved. As for the transporters, two cellobextrin transporters were characterized from *Neurospora crassa*: cellobextrin transporter 1 (CDT1) is a symporter with higher cellodextrin uptake activity (V_max), while cellobextrin transporter 2 (CDT2) is a facilitator with much lower activity (Galazka et al., 2010). Recent work on improving cellobextrin fermentation performance mainly focused on CDT1 (Du et al., 2012; Erikzen et al., 2013a; Fox et al., 2012; Ha et al., 2011a,b, 2013a; Li et al., 2010; Oh et al., 2013; Yuan and Zhao, 2013), because of the higher transporter activity and better cellobextrin fermentation performance under aerobic or oxygen-limited conditions. However, CDT1 is not optimal for anaerobic fermentation, a preferred process for industrial applications, since energy (ATP) consumption is coupled to cellobextrin uptake. On the contrary, the facilitator nature of CDT2 endows the energetic benefits under anaerobic conditions, due to no ATP consumption for cellobextrin uptake. Therefore, CDT2 has the potential to develop a better system for industrial production of cellulosic biofuels, albeit cellobextrin fermentation may be limited by the low transporter activity.

To overcome the main limitation of CDT2, we sought to increase its cellobextrin uptake activity using a directed evolution strategy. Although directed evolution has been successfully used to improve the activity, specificity, and stability of industrially important enzymes (Cobb et al., 2012, 2013; Luetz et al., 2008), its utilization in membrane-bound protein engineering is only limited to a few applications, such as the light-transducing protein bacteriorhodopsin (Hillebrecht et al., 2004; Wise et al., 2002), bacterial efflux pumps (Bokma et al., 2006; Foo and Leong, 2013), and xylose transporters in both *Escherichia coli* (Ren et al., 2009) and *S. cerevisiae* (Young et al., 2012). In this study, directed evolution was used to increase the cellobextrin uptake activity of CDT2, which resulted in improved cellobextrin fermentation under anaerobic conditions. After three rounds of directed evolution, the cellobextrin consumption rate and ethanol productivity were increased by 4.0- and 4.4-fold, respectively, under high cell density fermentation conditions. The evolved transporters were characterized for their relative expression level and specific cellobextrin uptake activity. The energetic benefits of the facilitator were evaluated by comparing cellobextrin fermentation performance of CDT1, CDT2, and CDT2 mutants under aerobic and anaerobic conditions, respectively.

Materials and Methods

Strains, Media, and Cultivation Conditions

*E. coli* strain DH5α (Life Technologies, Grand Island, NY) was used to maintain and amplify plasmids. *S. cerevisiae* INVSc1 strain (Life Technologies) was used as the host for homologous recombination based cloning and cellobextrin fermentation. Yeast strains were cultivated in complex medium consisting of 2% peptone and 1% yeast extract supplemented with either 2% glucose (YPD) or 2% cellobextrin (YPc). Recombinant strains were grown on synthetic complete medium consisting of 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 0.07% amino acid drop out mix without leucine (CSM-Leu, MP Biomedicals, Solon, OH), supplemented with 2% glucose (SCD-Leu) or 2% cellobextrin (SCC-Leu). *E. coli* strains were cultured at 37°C in Luria-Bertani broth containing 100 μg/mL ampicillin. *S. cerevisiae* strains were cultured at 30°C and 250 rpm for aerobic growth, and 30°C and 100 rpm in un-baffled shaker flasks for oxygen limited fermentation. For anaerobic fermentation, anaerobic culture tubes with butyl rubber stoppers and aluminum seals (Chernglass Life Sciences, Vineland, NJ) were vacuumed and purged with nitrogen to remove the residual oxygen and 420 mg/L Tween-80 and 10 mg/L ergosterol were supplemented as anaerobic growth factors (Verduyn et al., 1990). Tween-80 and ergosterol were dissolved in ethanol, and the supplementation resulted in around 0.5–1 g/L ethanol in the fermentation broth. Go Taq DNA polymerase for error-prone PCR was purchased from Promega (Madison, WI). All restriction enzymes, Q5 High Fidelity DNA polymerase, and the *E. coli*—*S. cerevisiae* shuttle vectors, pRS415 and pRS425, were purchased from New England Biolabs (Ipswich, MA). All chemicals were purchased from either Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

DNA Manipulation

The yeast homologous recombination based DNA assembler method (Shao et al., 2009) was used to construct recombinant plasmids and a library of CDT2 mutants. Briefly, polymerase chain reaction (PCR) was used to generate DNA fragments with homology arms at both ends, which were purified with a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and co-transformed along with the linearized backbone into *S. cerevisiae*. Oligonucleotides used in this study are listed in Table S1. To facilitate the creation of a library of CDT2 mutants, a helper plasmid with the functional elements *PYK1p*-NCU00130-*ADH1t*-TEF1p–*Pssl*-PGK1 cloned into pRS415 was constructed, named CDT-Eng-H hereafter (Fig. S1). NCU00130 is an *N. crassa* gene encoding β-glucosidase (BGL) and expressed intra-cellularly in *S. cerevisiae*. The helper plasmid was linearized by *Psl* digestion to allow the insertion of different cellodextrin transporters or a library of CDT2 mutants. NCU00801, NCU00809, and NCU08114 were cloned from pRS425-801, pRS425-809, and
pRS425-8114 constructed in our previous studies (Li et al., 2010), respectively, using primers CDT-F and CDT-R. To fuse enhanced green fluorescent protein (eGFP) at the C-terminus of the cellodextrin transporters with a Gly-Ser-eGFP-F2 and CDT2-eGFP-R2 to amplify the eGFP fragment, respectively, which were then cloned into the linearized CDT-Eng-H using the DNA assembler method. To confirm the correct clones, yeast plasmids were isolated using a Zymoprep Yeast Plasmid Miniprep II Kit (Zymo Research, Irvine, CA) and re-transformed into E. coli DH5α competent cells. Plasmids were isolated using a QiAprep Spin Miniprep Kit (Qiagen) and confirmed using both diagnostic PCR and DNA sequencing. All plasmids and strains used in this study are summarized in Table I. Yeast strains were transformed using the LiAc/SS carrier DNA/PEG (Gietz and Schiestl, 2007) method, and transformants were selected on SCD-Leu plates.

### Construction and Screening of a CDT2 Mutant Library

Error-prone PCR (Rubin-Pitel and Zhao, 2006) was used to create a library of CDT2 mutants, which was then cloned into linearized CDT-Eng-H. The concentration of MnCl₂ was adjusted to keep the mutation rate around 3 bp per kb (4–5 bp per gene), which was confirmed by DNA sequencing. After heat shock, a small amount of the transformant culture (0.1%) was spread onto an SCD-Leu plate to determine the library size. Under optimal conditions, 500 ng for each DNA fragment, a library ranging from 10⁵ to 10⁶ transformants could be obtained. The rest of the transformants were diluted appropriately, and around 10⁴ transformants were spread onto SCC-Leu agar plates. SCC plates were used for initial library screening, since the background growth of yeast cells on YP plates could interfere with the characterization of the size of the colonies. After incubation at 30°C for 3 days, many large colonies appeared and the top 60 colonies were picked and inoculated to 3 mL YPC liquid medium in culture tubes and samples were taken at 24, 28, and 32 h after inoculation to determine the cell density and specific growth rate. The top 12 mutants with either the highest cell density or specific growth rate were selected and the plasmids were extracted and re-transformed into fresh yeast cells to eliminate host adaptation. After re-transformation, the selected mutants

### Table I. Strains and plasmids used in this study.

<table>
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<tr>
<th>Name</th>
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<td>CDT1-eGFP</td>
<td>CDT1-eGFP inserted into CDT-Eng-H</td>
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were further confirmed using flask fermentation under oxygen limited conditions (10 mL YPC medium in 50 mL un-baffled flask) and samples were taken at 24, 28, 32, and 36 h after inoculation to measure cell density, cellobiose consumption, and ethanol productivity. The top 3 mutants with the highest cellobiose consumption and ethanol production rates were selected for final characterization under anaerobic conditions. The anaerobic tube was filled with 10 mL YPC medium and sample was taken every day until all sugar was consumed. In the whole process of library screening, seed cultures were grown in 3 mL SCD-Leu medium under aerobic conditions for 36 h in 14 mL culture tubes, and inoculated into YPC at an initial OD_{600} of 0.05. Iterative rounds of directed evolution were carried out until no further improvement was observed.

High Cell Density Fermentation

A single colony from the newly transformed plate was inoculated into 3 mL SCD-Leu medium, and cultured under aerobic conditions for 36 h. Then 1 mL seed culture was used to inoculate 15 mL YPC medium in a shake flask and cultured for an additional 24 h. The late-log phase cells were harvested and washed twice in double distilled water (ddH₂O), and inoculated into 10 mL YPC medium at an initial OD_{600} of 10 in a 20 mL anaerobic culture tube. Cellobiose fermentation was performed under anaerobic conditions and samples were taken every 3–4 h after inoculation until all cellobiose was consumed.


Cellobiose transport assay was performed using the oil-stop method as described previously (Galazka et al., 2010). A single colony of a yeast strain expressing a celldextrin transporter fused to eGFP was pre-grown in SCD-Leu medium for around 36 h, which was then inoculated into 10 mL fresh YPC medium. Mid-log phase cells cultured under oxygen-limited conditions were harvested and washed three times with the assay buffer (30 mM MES-NaOH and 50 mM ethanol, pH 5.5), and resuspended to an OD_{600} of 20. The uptake assay was initiated by adding 50 µL cells into 50 µL [^3]H]-cellobiose (Moravek Biochemicals, Brea, CA) layered over 100 µL silicone oil. Cells were centrifuged for 1 min at 15,000 rpm to stop the reaction after incubating with [^3]H]-cellobiose for 10, 20, 40, and 80 s, respectively. Then the tubes were frozen in ethanol/dry ice and the bottom fraction with cell pellets was collected and solubilized in 1 mL NaOH (0.5 M) overnight. Half of the supernatant was transferred into a scintillation vial and 3 mL ScintiSafe® Econo 1 Cocktail (Fisher Scientific) was added to read counts per minute (CPM) by a Beckman LS6500 scintillation counter (Beckman Coulter, Brea, CA). The maximum uptake rate (V_{max}) was determined by measuring the initial rate of cellobiose uptake at the concentration of 400 µM, which is about 100 fold higher than the K_m of CDT2. The value of V_{max} was normalized to both cellular level (cell density) and protein level (protein expression level as quantified by the eGFP fluorescence intensity).

Confocal Microscope Imaging

Yeast cells expressing eGFP tagged celldextrin transporters were cultured in YPC. A small droplet of mid-log phase cells were transferred onto a piece of cover glass and the fluorescence images were taken using a Zeiss LSM 700 Confocal Microscope (Carl Zeiss Microscopy, Thornwood, NY).

Analytical Methods

Cell growth was determined by measuring the absorbance at 600 nm using a Biotek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT), and eGFP fluorescence intensity was measured using a Tecan Safire® microplate reader (San Jose, CA) at 488 ± 5 and 509 ± 5 nm wavelengths for excitation and emission, respectively. Cellobiose and ethanol were quantified using Shimadzu HPLC (Columbia, MD) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) and Shimadzu RID-10A refractive index detector. The column was kept at 65°C and 0.5 mM sulfuric acid solution was used as a mobile phase at a constant flow rate of 0.6 mL/min. Each data point represents the mean of at least duplicates.

Prediction of Transporter Topology on Cellular Membrane

The topology of CDT2 on the cellular membrane was predicted using the HMMTOP online tool (http://www.enzim.hu/hmmtop/) (Tusnady and Simon, 2001), and the predicted result was visualized by the TMRPres2D tool (http://bioinformatics.biol.uoa.gr/TMRPres2D/) (Spyropoulos et al., 2004).

Results

Development of a Colony Size Based Screening Method for CDT Engineering

Owing to the important role of the sugar utilization pathway on cellular metabolism, the activity of the fermenting pathway can be coupled to the growth rate on a specific sugar, such as cellobiose and xylose. Therefore, the enrichment or adaptive evolution methods were commonly used to engineer efficient sugar fermenting strains (Cakar et al., 2012). Despite the success in constructing efficient cellobiose (Ha et al., 2013a,b) or xylose (Kim et al., 2013) fermenting yeasts, the improved phenotypes are often found to be related to the modification of the host genome rather than the engineered pathway, and it is rather difficult to figure out the molecular mechanisms of the random modifications. To minimize the impact of host adaptation, a colony size based screening method was developed in our group to engineer efficient sugar utilization pathways (Du et al., 2012; Yuan and Zhao, 2013). Sugar uptake is the first step of cellular
metabolism, and it is generally assumed that transporter is rate-limiting in the whole fermentation pathway (Eriksen et al., 2013a; Ha et al., 2013a; Jojima et al., 2010; Young et al., 2012). Therefore, the colony size based screening method was also applied to engineer the cellobiose transporter. Before performing directed evolution, this method was characterized using three N. crassa cellodextrin transporters with different activities, including NCU00801 (CDT1), NCU00809, and NCU08114 (CDT2) (Galazka et al., 2010). In accordance with the previous work (Ha et al., 2011a; Li et al., 2010), CDT1 performed the best in cellobiose fermentation, followed by CDT2 and then NCU00809, under aerobic conditions. As described in Figure 1, the control strain containing BGL but not CDT (CDT-Eng-H) only showed background growth (strain with the empty vector pRS415) on cellobiose, indicating that BGL was expressed intracellularly and the cellodextrin transporter activity was necessary to endow growth. Thus, different growth rates on cellobiose were resulted from the varied sugar uptake activities of CDT1, NCU00809, and CDT2. After spreading onto a cellobiose plate, the colony size was correlated with the growth rate in liquid medium, and therefore the cellobiose uptake activity (Fig. 1). These results also confirmed that CDT2 was rate-limiting in cellobiose fermentation under the screening conditions.

CDT2 Engineering Via Directed Evolution

Using the colony size based screening method, a general protocol for CDT2 engineering was developed (Fig. S2). For easier operation and based on the assumption that oxygen level cannot significantly change the activity of a facilitator, the initial screening steps were carried out under aerobic or oxygen-limited conditions, and only the final step of confirmation was performed under anaerobic conditions. In the first round of directed evolution, the top 2 mutants were found to carry the same mutations, Q207H/F209I/N311H (HIH), which validated the developed protocol for cellobiose transporter engineering. The role of these mutations in cellobiose fermentation was analyzed by creating all different combinations of the mutations including Q207H, F209I, N311H, Q207H/F209I (HI), Q207H/N311H (HH), and F209I/N311H (IH). As shown in Figure S3, variants HH, HIH, N311H, and Q207H grew faster than the wild-type CDT2 (WT), while variants HI and F209I grew slower than WT. Accordingly, it could be concluded that Q207H and N311H were beneficial mutations, whereas the F209I mutation impaired the cellobiose uptake activity.

Using the mutants obtained in the second round of directed evolution, the cellobiose fermentation performance was compared under aerobic, oxygen-limited, and anaerobic conditions. In agreement with our assumption that the activity of a facilitator is not significantly changed by the oxygen level, the growth and cellobiose consumption rates of these mutants were closely correlated under different conditions. As shown in Figure 2, the mutants that consumed cellobiose faster under oxygen-limited conditions also showed improved cellobiose fermentation under anaerobic conditions (HH > HIH > Q207H > WT). After validating

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Development of a colony size based screening method for CDT engineering. Different CDTs were introduced into CDT-Eng-H, a plasmid containing the β-glucosidase (BGL) from N. crassa. The colony size on SCC-Leu agar plate (middle panel) was confirmed to be positively related to the growth rate in SCC-Leu liquid medium (left panel), as well as the cellobiose uptake activity (right panel). Bars on the right panel represented the relative cellobiose uptake activity, with CDT1 activity set to 100%.

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our developed protocol for CDT engineering, a third round of directed evolution was carried out using the best mutant HH as the template. Similarly, mutants with increased cellobiose consumption rate were obtained, and DNA sequencing revealed that the best mutant harbored only one additional mutation I505T, named HHT hereafter. The fourth round of directed evolution did not result in mutants with significantly improved cellobiose fermentation performance (data not shown), and no further directed evolution of CDT2 by error-prone PCR was attempted.

Subsequently, the cellobiose fermentation performance (sugar consumption rate, ethanol productivity, and yield) of WT and the best mutants obtained in the first (HIH), second (HH), and third (HHT) round of directed evolution was compared under anaerobic conditions. Using the same conditions as library screening, the specific growth rate, sugar consumption rate, and ethanol productivity were increased by 2.6-, 3.0-, and 5.5-fold, respectively (Fig. S4). If a high cell density was used for anaerobic fermentation, the strain containing the evolved transporter was able to consume 20 g/L cellobiose in less than 8 h, while the wild-type transporter required more than 30 h to use up the same amount of cellobiose. The best mutant (HHT) conferred an increase of 4.0- and 4.4-fold for the cellobiose consumption rate and ethanol productivity, respectively (Fig. 3 and Table SII). Moreover, the overall ethanol yield, which is another important parameter for cost-effective production of cellulosic biofuels, was improved by more than 25%.

**Mutant Analysis by Site-Directed Mutagenesis and Saturation Mutagenesis**

After three rounds of library construction and screening, the best mutant obtained via directed evolution carried three mutations, Q207H/N311H/I505T (HHT). To investigate the role of these mutations in cellobiose fermentation, site-directed mutagenesis was carried out to construct the additional single mutant I505T and the combined double mutants including Q207H/I505T and N311H/I505T. Cellobiose fermentation performance of all these variants was compared with WT and the triple mutant HHT under oxygen-limited conditions. As shown in Figure S5, all the single mutants grew faster than the wild-type (N311H > I505T > Q207H > WT), indicating the beneficial mutations at these three sites. In addition, the mutations were additive in terms of cellobiose fermentation performance, meaning that the double mutants grew faster than the single mutants and slower than the triple mutant. Although the mutation I505T
increased the cellobiose uptake rate more than Q207H, HH worked the best among the double mutants (HH > N3117H/I505T > Q207H/I505T) for cellobiose fermentation.

Saturation mutagenesis (McLachlan et al., 2008) libraries were then constructed at the beneficial mutation sites individually (Q207, N311, and I505) using degenerate primers (Table SI). Following the same library screening process, top 5 clones showing the fastest growth on cellobiose were chosen and their corresponding nucleotide sequences were verified by DNA sequencing. At the I505 site, no clones grew faster than I505T; at the Q207 site, four out of five clones possessed the same amino acid change (Q207T), although their nucleotide sequences were different; at the N311 site, all five clones shared the same mutation N311G (Table SIII). In hope of further improving the cellobiose fermentation performance, the new mutations obtained by saturation mutagenesis were combined to construct Q207H/N311G/I505T (HGT), Q207T/N311H/I505T (THT), and Q207T/N311G/I505T (TGT). The cellobiose fermentation performance of these new triple mutants was compared with Q207H/N311H/I505T (HHT) under anaerobic conditions. Unfortunately, none of them showed improved cellobiose fermentation performance (Table II). Thus, no further engineering of CDT2 was attempted.

Characterization of the Evolved CDT2 Mutants

To investigate the role of mutations in cellobiose uptake activity, we first determined the relative expression levels of CDT2 and its mutants. As shown in Figure 4A, the cytoplasmic membrane localization of the transporter-eGFP fusion proteins was confirmed by confocal microscope imaging. Therefore, the relative expression levels of the cellobiose uptake activity and the cellobiose fermentation performance, the new mutations obtained by saturation mutagenesis were combined to construct Q207H/N311G/I505T (HGT), Q207T/N311H/I505T (THT), and Q207T/N311G/I505T (TGT). The cellobiose fermentation performance of these new triple mutants was compared with Q207H/N311H/I505T (HHT) under anaerobic conditions.

Table II. Anaerobic cellobiose consumption rate of CDT2 mutants obtained by site-directed mutagenesis and saturation mutagenesis.

<table>
<thead>
<tr>
<th>CDT2 mutants</th>
<th>Cellobiose consumption rate (g/L·h)</th>
<th>Ethanol productivity (g/L·h)</th>
<th>CDT2 mutants</th>
<th>Cellobiose consumption rate (g/L·h)</th>
<th>Ethanol productivity (g/L·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.054 ± 0.002</td>
<td>0.011 ± 0.002</td>
<td>I505T</td>
<td>0.069 ± 0.005</td>
<td>0.015 ± 0.004</td>
</tr>
<tr>
<td>Q207H</td>
<td>0.058 ± 0.002</td>
<td>0.013 ± 0.001</td>
<td>HHT</td>
<td>0.160 ± 0.004</td>
<td>0.060 ± 0.001</td>
</tr>
<tr>
<td>Q207T</td>
<td>0.098 ± 0.012</td>
<td>0.023 ± 0.009</td>
<td>HGT</td>
<td>0.140 ± 0.006</td>
<td>0.052 ± 0.004</td>
</tr>
<tr>
<td>N311H</td>
<td>0.072 ± 0.011</td>
<td>0.019 ± 0.002</td>
<td>THT</td>
<td>0.130 ± 0.003</td>
<td>0.049 ± 0.001</td>
</tr>
<tr>
<td>N311G</td>
<td>0.092 ± 0.007</td>
<td>0.021 ± 0.002</td>
<td>TGT</td>
<td>0.120 ± 0.002</td>
<td>0.045 ± 0.002</td>
</tr>
</tbody>
</table>

Cells were pre-cultured in SCD-Leu and inoculated into fresh YPC with an initial OD₆₀₀ of 0.05.
correlated with the fermentation performance, that is, higher activity resulted in increased specific growth rate, cellobiose consumption rate, and ethanol productivity (Figs. 3 and 4C). By normalizing the transporter activity to the relative protein expression level, it was found that the specific activity of HH and HHT were nearly the same, indicating that Q207H and N311H increased the specific uptake activity, while I505T contributed to increased transporter expression level (Fig. 4D). Considering the role of I505T in cellobiose uptake activity and cellobiose fermentation, it was assumed that higher level expression of the transporter protein would further improve cellobiose fermentation performance. Currently, the expression of CDT2 was under the control of TEF1 promoter (TEF1p), which was shown to be the strongest constitutive promoter in S. cerevisiae (Sun et al., 2012). Therefore, the cellobiose utilization pathway was subcloned into a 2μ based multiple copy plasmid to achieve higher level expression of the celldextrin transporter. Unfortunately, the switch to a multiple copy plasmid led to dramatically extended lag phase for cellobiose consumption (Fig. S6), probably due to the burden of maintaining the plasmid with increased copy number and/or the cytotoxicity of overexpressing membrane proteins (Kai et al., 2010; Lian et al., 2009b).

**Energetic Benefits of the Evolved CDT2 Mutants on Anaerobic Cellobiose Fermentation**

To demonstrate the energetic benefits of facilitators for anaerobic cellobiose utilization, cellobiose fermentation of CDT1, CDT2, and CDT2 mutants were compared under aerobic and anaerobic conditions, respectively. In agreement with previous studies (Ha et al., 2011a; Li et al., 2010), CDT1 showed the best cellobiose fermentation performance under aerobic conditions (Fig. S7) due to its highest cellobiose uptake activity (Fig. 4C). Under anaerobic conditions, although CDT1 still performed better than WT CDT2, the CDT2 mutants showed significantly improved cellobiose consumption and ethanol production (Fig. 3). In other words, the evolved CDT2 was more energetically favorable than CDT1 for anaerobic cellobiose uptake and metabolism. These results indicated that the energetic benefits of WT

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**Figure 4.** Characterization of the expression level and cellobiose uptake activity of CDT1, CDT2, and CDT2 mutants. The expression level of celldextrin transporters were quantified by fluorescence intensity, and sugar uptake assay was performed using [3H]-Cellobiose. (A) Confocal microscope image of yeast cells expressing CDT2-eGFP. (B) Relative expression levels of celldextrin transporters in YPC. NC is the control strain (WT) without eGFP tagging. (C) For the transporter activity at cellular level, cellobiose uptake assay was performed at OD600 1. (D) Cellobiose uptake activity at the protein level was normalized to the relative transporter expression level, with the specific cellobiose uptake activity of WT-eGFP set to 1.
CDT2 as a facilitator was limited by its low cellobiose uptake activity, which could be overcome by directed evolution of this cellodextrin transporter. Compared with CDT1, the evolved CDT2 (HHT) showed 2.5- and 2.9-fold increase in anaerobic cellobiose consumption rate and ethanol productivity, respectively.

**Discussion**

Intense research has been focused on producing fuels and chemicals from lignocellulose biomass (Du et al., 2011; Hahn-Hagerdal et al., 2007; Jang et al., 2012). To make the cellulosic biofuel fermentation process economically feasible, the host should be able to consume a mixture of sugars efficiently and simultaneously. However, the presence of glucose repression led to sequential utilization of mixed sugars and low productivity of biofuels. Three general strategies are proposed to overcome glucose repression to allow sugar co-utilization (Kim et al., 2012): the construction of glucose-derepressed yeasts by perturbing the glucose sensing and signaling network, the introduction of xylose specific transporters, and the introduction of a cellobiose utilization pathway including a cellodextrin transporter and a β-glucosidase. Due to the complexity of the regulatory system, yeast strains without glucose repression on xylose metabolism have never been obtained (Kim et al., 2012; Roca et al., 2004). As for xylose transporters, they suffer from either low uptake activity or low specificity (Du et al., 2010; Jojima et al., 2010). Even after extensive cloning and engineering of xylose transporters, efficient co-fermentation of glucose and xylose has not been achieved either (Young et al., 2012). On the contrary, the cellobiose utilization strategy has been proven to be able to overcome glucose repression, allowing co-fermentation of cellobiose/xylose (Ha et al., 2011a; Li et al., 2010), and cellobiose/galactose (Ha et al., 2011b) efficiently. Another advantage of the cellobiose utilization strategy is the application in consolidated bioprocessing (simultaneous saccharification and fermentation,SSF), because the utilization of cellobiose instead of glucose can eliminate the requirement of β-glucosidase supplementation in current cellulase cocktails (Fox et al., 2012; Galazka et al., 2010; Jin et al., 2013).

Protein engineering, including the directed evolution approach, the knowledge-driven rational design approach, and a hybrid approach combing directed evolution and rational design, has been widely used to improve the performance of a wide variety of enzymes and pathways (Eriksen et al., 2013b; Wen et al., 2009). Although there are numerous protein engineering examples (Cobb et al., 2012, 2013), there are only a few examples of engineering membrane proteins, especially transporters (Bokma et al., 2006; Foo and Leong, 2013; Ren et al., 2009; Young et al., 2012). It is rather challenging to engineer membrane proteins because they are associated with the membranes. Rational design is hindered by the lack or limited availability of high-resolution structural information (Hovijititra et al., 2009; Lian et al., 2009b). Directed evolution is limited by the difficulty in determining the activity of membrane proteins, let alone the development of a high-throughput screening system. As the bridge between intra- and extra-cellular environments, membrane proteins are mainly involved in the transduction of extra-cellular signals, transportation of substances through membrane, and generation of energy, whose activities are difficult or labor-intensive to determine (Kai et al., 2010; Lian et al., 2009a). Fortunately, sugar transporters are essential to initiate the cellular metabolism, and a high throughput screening method based on cell growth rate can be readily developed. Engineering of xylose transporters was carried out in a host with the xylose utilization pathway integrated to the chromosome and all hexose transporters inactivated (Young et al., 2012). *S. cerevisiae* lacked an endogenous cellobiose uptake system, and a colony size based screening system was developed for cellodextrin transporter engineering (Figs. 1 and S2). Using this high-throughput screening system, cellobiose fermentation performance (Figs. 3 and S4) and cellobiose uptake activity (Fig. 4C) were significantly improved after three rounds of directed evolution. Due to the lack of 3-D structure of celloextrin transporters, the HMMTOP online tool (Tusnady and Simon, 2001) and the TMProS2D tool (Spyropoulos et al., 2004) were used to predict and visualize the secondary structure of CDT2. Based on the predicted topology, I505 was located in the last intra-cellular loop, and Q207 and N311 were located in the sixth and seventh transmembrane helixes (Fig. 5). The predicted positions of these mutations were consistent with their corresponding roles: the residue in the intra-cellular loop might not be related to cellobiose uptake directly, while the inner membrane located residues might be involved in the cellobiose channel formation and affect the specific uptake activity. Previous work on transporter engineering revealed that the mutation in the intra-cellular loops could increase the sugar uptake activity (Eriksen et al., 2013a; Young et al., 2012), which might result from increased transporter expression levels. The saturation mutagenesis results also supported the involvement of Q207 and N311 in the formation of the cellobiose channel. The change to an amino acid residue with smaller size (Q207T or N311G) might enlarge the channel and facilitate cellobiose uptake (Table II). Q207 and N311 mutations were combined to construct CDT2 mutants with larger cellobiose channel, which further improved cellobiose uptake and cellobiose fermentation performance. Nevertheless, HHT still performed the best for cellobiose fermentation among all mutants constructed in this study, probably due to the synergy of the two histidine residues on cellobiose uptake. It would be interesting to test the activity of HGT, THT, and TGT towards other cello-oligosaccharides, such as cellotriose and cellotetrose. Although their cellobiose uptake activities were lower than that of HHT, the enlarged channel might benefit the uptake of longer oligosaccharides.

Recently, a bacterial cellobiose phosphorylase together with a highly functional CDT1 mutant were introduced into *S. cerevisiae* and confirmed to perform better than the hydrolytic pathway under low oxygen or acetate supplemented conditions.
(Ha et al., 2013a). The energy requirement of CDT1 for cellobiose uptake could be to some extent compensated by the phosphorolytic pathway under stress conditions, owing to the energetic benefits of cellobiose phosphorolysis. In the hydrolytic pathway, cellobiose is cleaved by \( \beta \)-glucosidase to generate two glucose molecules, which need to be phosphorylated by 2 ATP molecules (per cellobiose) to enter glycolysis. In the phosphorolytic pathway, cellobiose is cleaved by phosphorylase to release one glucose molecule and one glucose-1-phosphate molecule, and only one ATP molecule (per cellobiose) is consumed to initiate fermentation (Zhang and Lynd, 2004). Despite the energetic benefits, a CDT1 mutant with significantly improved uptake activity was necessary to provide a “push” driving force to enable efficient cellobiose fermentation (Ha et al., 2013a). Therefore, a cellobiose pathway combining the engineered CDT2 with cellobiose phosphorylase may have the maximized energetic benefits, and further improve cellobiose fermentation performance under anaerobic conditions.

In summary, the cellobiose uptake activity of CDT2 was increased via directed evolution to improve anaerobic cellobiose fermentation performance, including the specific growth rate, cellobiose consumption rate, ethanol productivity, and ethanol yield. In addition, the engineered CDT2 showed energetic benefits as a facilitator for cellobiose fermentation under anaerobic conditions. For the best mutant (HHT) obtained after three rounds of directed evolution, the two mutations located in transmembrane helices conferred a 1.8-fold increase in the specific cellobiose uptake activity, while the mutation in the intra-cellular loop was determined to increase the transporter expression level rather than the specific activity. To our knowledge, this is the first report of using directed evolution to increase the activity of a cellodextrin transporter and the productivity of cellulosic biofuels under anaerobic conditions. In addition, the directed evolution strategy developed in this study can be extended to engineer other sugar transporters, such as xylose and arabinose transporters (Gardonyi et al., 2003), which are considered rate-limiting for cellulosic biofuel production.

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References


**Supporting Information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.