Supporting Online Material for

**Cellodextrin Transport in Yeast for Improved Biofuel Production**

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Transporter and β-glucosidase orthologues

GenBank accession numbers or Joint Genome Institute (JGI) protein ID (PID) numbers for celloextrin 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.

GenBank accession numbers for celloextrin hydrolases that are orthologues of NCU00130 (named gh1-1; in glycosyl hydrolase family GH1) are as follows: T. melanosporum, CAZ82985.1; A. oryzae, BAE57671.1; P. placenta, EED81359.1, and P. chrysosporium, BAE87009.1. The other organisms that contain celloextrin transporter orthologues contain genes in the GH3 family predicted to be intracellular β-glucosidases (S3, S4), as follows: Kluyveromyces lactis, CAG99696.1; Laccaria bicolor, EDR09330; Clavispora lusitaniae, EEQ37997.1; and Pichia stipitis, ABN67130.1.
Materials and Methods

Strains

The yeast strain used in this study was YPH499 (S5), which has the genotype: MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1. It was grown in YPD media supplemented to 100 mg/L adenine hemisulfate. Transformed strains (S6) 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) (S7) and include WT (FGSC 2489) and two cellobiose transporter deletion strains (FGSC 16575, ΔNCU00801.2 and FGSC 17868, ΔNCU08114.2 (S8)).

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, ATATATGAGCTCGTGAGTAAGAAAAGAGTGAGGAACTATC and ATATATACTAGTTTTTATATTTGTGAAAAAGTAGATAATTACTTCC (In all primers above and below, restriction sites are underlined). NCU00801 with a C-terminal Myc-tag and optimized Kozak sequence (S9) was then inserted between BamHI and EcoRI using the primers,
ATGGATCCAAAAATGTGTCGCTCAGGCTCC and
ATGAATTCTACAAATCTTTCCAGAAATCAATTTTTTGTTCAGCAACGATAGC
TTCGGAC, and NCU08114 with a C-terminal Myc-tag and optimized Kozak
sequence was inserted between SpeI and Clai using the primers,
ATACTAGAAAAATGGGCATCTTCAACAAGAAGC and
GCATATCGACTACAAATCTTTCCAGAAATCAATTTTTTGTTCAGCAACAGAC
TTGCCCTCATG. To make GFP fusions, superfolder GFP (S10) with an N-
terminus 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,
TATTAAATCGATGGTATGGTATGGTATGGCAAGGGCGAGGAG and
TATTAAGTCGACCTACTTGTACAGCTCATTCCATGCC. Transporters were then
fused to GFP as follows: NCU00801 was inserted between BamHI and EcoRI
using the primers, GCATGGATCCATGCTCTCACGGCTCC and
TATAATGAATTCAACAGATAGCTTCGGAC, and NCU08114 was inserted
between SpeI and EcoRI using the primers,
TATTAAACTAGTATGGGCATCTTCAACAAGAAGC and
TTATAAGAACCAACAGACTTGCCCTCATG.
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,
GCATACTAGAAAAATGTGTCGCTTCTCTCTTCTTTTCAGGATTTTCTCT and
ATACTGCAATTAGATGATGATGATGATGGTCTTCTTTGATCAAAGAGTCA
AAG. All constructs included the Cyc transcriptional terminator between XhoI and KpnI. All *N. crassa* genes were amplified by PCR from cDNA 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 celloextrins, 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 O.D. (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 O.D. 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 O.D. (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 (Fig. S9), 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 Orthologues

Amino acid sequences of orthologues of cdt-1 and cdt-2 were obtained from online databases. Multiple sequence alignments were performed using T-
Coffee (S11). A maximum likelihood phylogeny was determined using PhyML version 3.0 (S12) 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 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 O.D. 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 concentration in the filtrate was 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 (S8) were acquired from the FGSC (S7), 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 (S8) of NCU00801 (FGSC 16575) and NCU08114 (FGSC 17868) were acquired from the FGSC (S7), 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$_2$O 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 O.D. (600 nm) of 0.8 – 1.2, using a 100x 1.4 NA 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.


Transport assays were performed using a modification of the oil-stop method (S13). Yeast strains expressing either cdt-1 or cdt-2 fused to GFP were grown to an O.D. (600 nm) of 1.5 – 3.0 in selective media, washed 3x with ice cold assay buffer (30 mM MES-NaOH [pH 5.6] and 50 mM ethanol), and resuspended to an O.D. 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 [³H]-cellobiose uptake over 3 minutes for cellobiose concentrations between 0.5 and 200 µM. \( V_{\text{max}} \) 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®. \( V_{\text{max}} \) values were normalized for differences in transporter abundance by measuring the GFP fluorescence from 100 µL of cells at O.D. 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 [³H]-cellobiose over 20 s in the presence of 250 µM of the respective competitors.

Measurement of \( \beta \)-glucosidase activity in culture media

To assay for contaminating \( \beta \)-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 ddH₂O and resuspended to an O.D. (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 O.D. (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 O.D. (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) (S16). 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, Fig. S5) 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 ddH₂O, and resuspended to an O.D. (600 nm) of 0.1 in 50 mL YNB plus the appropriate CSM and 2% (w/v) cellobiose. Cultures were grown in 250 mL unbaffled flasks at 30 °C, with shaking at 200 RPM. The change in O.D. (600 nm) was monitored by periodically removing samples.
Fig. S1. 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).
Fig. S2. Celloextrin 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 (*S*14) that hydrolyze celloextrins to glucose, which is subsequently taken up by monosaccharide transporters (*S*15). This alternate route of consumption leads to an underestimate of the celloextrin transport defect in these deletion lines.
Fig. S3. 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 S4

A

B

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**Fig. S4.** Growth of *S. cerevisiae* strains on glucose and cellobiose. (A) Growth of the strains on glucose using the Bioscreen-C™, for comparison to Figure 1B, 1C. Shown is the growth of yeast strains expressing *cdt*-1 (○), *cdt*-2 (▼), or no transporter (●). All strains express the β-glucosidase *gh1*-1. A representative experiment is shown. Growth rates from three independent growth experiments are as follows: *cdt*-1, 0.110 ± 0.014 hr⁻¹; *cdt*-2, 0.116 ± 0.003 hr⁻¹; no transporter, 0.131 ± 0.0002 hr⁻¹ (mean ± SD). (B) Growth of the strains on cellobiose in 250 mL flasks. Values represent the mean O.D. between two replicate cultures of yeast strains expressing the β-glucosidase *gh1*-1, *cdt*-1 or *cdt*-2, or a strain expressing *gh1*-1, but lacking any transporters. Approximate doubling times of yeast in these experiments is 3.2 hours (*cdt*-1) and 4.7 hours (*cdt*-2).
**Fig. S5.** Hydrolysis of cellobextrins 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.
Fig. S6. β-glucosidase activity in cell culture broth after growth experiments. (A) Final O.D. 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 O.D. 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.
**Fig. S7.** 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.
Fig. S8. Competition by celldextrins 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 [³H]-cellobiose transport. Substrates of CDT-1 or CDT-2 should decrease the [³H]-cellobiose transport rate by competing for binding. Values (average of three independent experiments ±SD) were normalized by setting the rate of [³H]-cellobiose transport without a competing sugar to 100.
References