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Research Article

RESEARCH ARTICLE

The *Lipomyces starkeyi* gene *Ls120451* encodes a cellobiose transporter that enables cellobiose fermentation in *Saccharomyces cerevisiae*

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One sentence summary: Putative sugar transporters Ls120459 from *Lipomyces starkeyi* and CdtG from *Penicillium oxalicum* confer growth on cellobiose when expressed in *Saccharomyces cerevisiae*.

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ABSTRACT

Processed lignocellulosic biomass is a source of mixed sugars that can be used for microbial fermentation into fuels or higher value products, like chemicals. Previously, the yeast *Saccharomyces cerevisiae* was engineered to utilize its cellodextrins through the heterologous expression of sugar transporters together with an intracellular expressed β-glucosidase. In this study, we screened a selection of eight (putative) cellodextrin transporters from different yeast and fungal hosts in order to extend the catalogue of available cellobiose transporters for cellobiose fermentation in *S. cerevisiae*. We confirmed that several *in silico* predicted cellodextrin transporters from *Aspergillus niger* were capable of transporting cellobiose with low affinity. In addition, we found a novel cellobiose transporter from the yeast *Lipomyces starkeyi*, encoded by the gene *Ls120451*. This transporter allowed efficient growth on cellobiose, while it also grew on glucose and lactose, but not cellotriose nor cellotetraose. We characterized the transporter more in-depth together with the transporter CdtG from *Penicillium oxalicum*. CdtG showed to be slightly more efficient in cellobiose consumption than Ls120451 at concentrations below 1.0 g/L. Ls120451 was more efficient in cellobiose consumption at higher concentrations and strains expressing this transporter grew slightly slower, but produced up to 30% more ethanol than CdtG.

Keywords: *Saccharomyces cerevisiae*; cellodextrin transporter; cellobiose fermentation; *Lipomyces starkeyi*; CdtG; sugar transporter

BACKGROUND

Due to its capacity to ferment sugars into ethanol the yeast *Saccharomyces cerevisiae* has been used extensively for the industrial production of bioethanol (Favaro, Jansen and van Zyl 2019). Traditionally, the main sources for these sugars are glucose rich hydrolysates from edible crops like corn or sugarcane, which are grown specifically for this purpose (Walker and Walker 2018). However, for economic and sustainability reasons it would be preferable to use materials coming from non-edible crops (Wyman 2007). The main components of these cellulosic feedstocks are cellulose, hemicellulose and lignin. Currently, the main strategy to release glucose from these plant cell wall components is to add large quantities of cellulase cocktails (Aditiya

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et al. 2016). Unfortunately, this is not very cost-efficient and it causes economic constraints in switching the ethanol production process to this type of carbon source (Favaro, Jansen and van Zyl 2019). In addition, native *S. cerevisiae* is unable to ferment the cellodextrins that are released by the cellulases and it requires additional β-glucosidase supplementation in order to produce enough glucose to ferment, thus increasing the economic limitations (Aditiya *et al.* 2016).

Therefore, there has been extensive research into creating *S. cerevisiae* strains that can metabolize sugar polymers like cellodextrins. In 2010, it has been reported that a minimal system for cellodextrin fermentation by *S. cerevisiae* consists of recombinant expression of a cellodextrin transporter, together with an intracellular β-glucosidase to hydrolyse the cellodextrins (Galazka *et al.* 2010). Galazka *et al* introduced the *Neurospora crassa* cellodextrin transporters CDT-1 or CDT-2 into *S. cerevisiae* and combined their expression with the intracellular expression of the β-glucosidase GH1–1 from the same mould (Galazka *et al.* 2010; Kim *et al.* 2014). The possibilities for this yeast system are especially interesting when considering the engineering successes for *S. cerevisiae* to metabolise pentose sugars like xylose, as this sugar is as well abundant in lignocellulosic hydrolysates. Currently, *S. cerevisiae* strains have been constructed containing heterologous xylose metabolism pathways so that they can use xylose as a carbon source (Kim *et al.* 2013). However, one of the main limitations is still the inhibition of xylose uptake by the presence of glucose; making the cells only ferment xylose after all glucose is consumed, thus decreasing the ethanol yield and productivity. Strains which have both the cellobiose and xylose assimilation pathways have shown to be able to ferment both carbon sources simultaneous, resulting in high ethanol yields and productivity (Li *et al.* 2010; Ha *et al.* 2011).

In the research to improve the *S. cerevisiae* strains capable of cellobiose fermentation, it was found that the hydrolytic GH1–1 enzyme could also be replaced by a β-glucosidase from a different host (Bae *et al.* 2014), or replaced by a phoshorylitic enzyme, for example a cellodextrin phosphorylase from *Saccharophagus degradans* (Ha *et al.* 2013a; Kim *et al.* 2018). On the other hand, the two *N. crassa* transporters have been studied extensively in order to improve their characteristics, for example using directed evolution approaches. For both of the cellodextrin transporters this has led to the discovery of more active or more stable mutants (Lian *et al.* 2014; Sen *et al.* 2016; Oh *et al.* 2017). After the discovery of *N. crassa* CDT-1 and CDT-2, more permease genes from different organisms were found with an ability to transport cellodextrins and related sugars in *S. cerevisiae*, for example *HXT2.4* from *Pichia stipitis* (Ha *et al.* 2013b), and *LAC12* from *Kluyveromyces lactis* (Sadie *et al.* 2011). Bae and colleagues screened a set of fungal cellobiose transporters from *Trichoderma reesei, Penicillium chrysogenum,* and *Thielavia terrestris,* together with different β-glucosidases (Bae *et al.* 2014). They report a combination with an improved cellobiose fermentation beyond the typical *N. crassa* CDT-1 and GH1–1 combination. These results indicate that there are still novel sugar transporters to be discovered with improved and different sugar transport characteristics that could be useful in the *S. cerevisiae* based production process.

Some of these novel sugar transporters have been found by characterizing the original host in unrelated processes (Martin and Russell 1987; Helaszek and White 1991; Maas and Glass 1991), sometimes using deletion strains to look for phenotypes. Often, the transporters that have been found were only studied shallowly in *S. cerevisiae* in order to confirm their transport function in an isolated system. For example, the cellodextrin transporters CdtC, CdtD, and CdtG from *Penicillium oxalicum,* for which only growth and cellobiose uptake was confirmed in *S. cerevisiae* (Li *et al.* 2013). In addition, the use of bioinformatics methods has led to the discovery of many putative sugar transporters. For example, Peng *et al.* found 86 putative sugar transporters in *Aspergillus niger* using an *in silico* approach (Peng *et al.* 2018). However, these transporters were not further characterized *in vivo* beyond their expression levels in *A. niger* when grown on various carbon sources.

In this study, we selected a set of (putative) sugar transporters for further characterization in *S. cerevisiae*. A total of five *A. niger* genes were selected from the study from *Peng et al* (2018), these genes were supplemented by CdtG from *P. oxalicum* (Li *et al.* 2013) and CltA from *Aspergillus nidulans* (Dos Reis *et al.* 2016). In addition, we included the gene *LIPSDRAFT120451* (from here on this gene is referred to as *Ls120451*) from the ascomycetous yeast *Lipomyces starkeyi* based on its sequence similarity to CDT-1 and CDT-2. We expressed the recombinant genes in an *S. cerevisiae* strain that cannot transport hexoses and that expresses the *N. crassa* β-glucosidase GH1–1 intracellularly. In this host, we analysed growth on solid and liquid media using a selection of carbon sources. Finally, we characterized the transporters Ls120451 and CdtG more in-depth for their sugar consumption and fermentation characteristics in *S. cerevisiae*.

RESULTS AND DISCUSSION

Selection of sugar transporter genes for analysis in S. cerevisiae

The sugar transporters CDT-1 and CDT-2 from *N. crassa* have been shown to be versatile in their sugar transport profile and are to date still two of the most efficient transporters for cellodextrins (Kim *et al.* 2014). Therefore, we used these two transporters as a model for our search for candidates for the screen of sugar transporters in *S. cerevisiae* (Table 1). We searched literature for (putative) cellobiose transporters that were uncharacterized or poorly characterized in *S. cerevisiae*. In addition, we looked for possible new candidates in organisms with interesting growth characteristics regarding their carbon source utilization.

Peng and colleagues published in 2018 a thorough *in silico* study of sugar transporters in the fungus *Aspergillus niger,* in which they described a number of putative cellodextrin transporters which phylogenetically clustered together with known cellodextrin and lactose transporters (including both CDT-1 and CDT-2) (Peng *et al.* 2018). From this group, we selected the genes *An14g01600*, *An16g06220*, *An08g09350*, *An13g03250*, and *An03g05320* for use in this study (Peng *et al.* 2018). Next, we selected the genes *CltA* from *A. nidulans* and *CdtG* from *P. oxalicum*. The first of these two genes was found to be a cellodextrin transporter by dos Reis *et al* (Dos Reis *et al.* 2016). The latter gene is part of a family of cellodextrin transporters found by Li and colleagues (Li *et al.* 2013). In their study, CdtG was clustered together with Lac12p from *K. lactis* and CDT-1, indicating that it might have interesting transport properties also beyond just cellobiose transport. Interestingly, CdtG was reported to be not that efficient in cellobiose transport compared to CdtC and CdtD from the same host (Li *et al.* 2013). The last putative cellobiose transporter sequence we selected has, to our knowledge, not yet been published nor investigated before. In 2012, the yeast *L. starkeyi* was shown to be able to efficiently ferment cellobiose and xylose to produce lipids (Gong *et al.* 2012). As there is a draft genome available of this yeast (Riley *et al.* 2016), we used the BLAST website to compare the genome sequences with the sequences of CDT-1 and CDT-2. This search pointed us to the gene *LIPSDRAFT120451*, which was marked as a putative

Table 1. Sugar transporters analysed in this study.

Source organism	Transporter name	Genebank accession number	Reference
Lipomyces starkeyi	Ls120451	ODQ69961.1	This study
Aspergillus niger	An14g01600	XP_0 014 00787.2	Peng *et al.* (2018)
	An16g06220	CAK46958.1	Peng *et al.* (2018)
	An08g09350	CAK96714.1	Peng *et al.* (2018)
	An13g03250	XP_0 013 96460.1	Peng *et al.* (2018)
	An03g05320	XP_0 013 90413.1	Peng *et al.* (2018)
Penicillium oxalicum	CdtG	AGN74922.1	Li *et al.* (2013)
Aspergillus nidulans	CltA	XP_681 616.1	dos Reis *et al.* (2016)

membrane protein and predicted to be a sugar transporter from the major facilitator superfamily (MFS). In general, the protein sequences of all the selected transporters are 25–35% identical to CDT-1 and 30–41% identical to CDT-2. All the sequences selected were predicted to localize to the plasma membrane according to a sequence based analysis using the Busca programme (http://busca.biocomp.unibo.it/) (Savojardo *et al.* 2018).

Next, we used the Geneious software to multiple align the selected sequences, together with a selection of 45 additional sugar transporter sequences from different yeast and fungi with different sugar transport specificities (Supplemental Table 1), followed by a phylogenetic tree analysis (Fig. 1). The major clades in the phylogenetic tree were grouping together by the main type of sugar that they transport, showing on the bottom half the transporters for glucose (red) and xylose (orange). The big group on the top consisted of transporters for cellobiose, cellodextrins, and lactose (blue), with finally a small group for maltose and maltotriose transporters on the right (green) (Fig. 1). The analysis clustered all eight of our selected transporters in the same clade with the transporters that have been known to transport disaccharides like lactose and cellobiose (transporters that were previously shown to transport cellobiose are marked with an $*$), and in some cases longer cellodextrins. Most of the transporters studied here clustered together with CDT-2 and other known cellobiose transporters. However, CdtG was more closely related to CDT-1 and lactose transporters, like lacpA and lacpB from *A. nidulans*, Lac12p from *K. lactis*, and Lac2p from *P. stipitis*. This could indicate that, in addition to cellobiose, CdtG might also be able to transport lactose, as for example CDT-1 and Lac12p are able to transport both cellobiose and lactose (Sadie *et al.* 2011; Liu *et al.* 2016).

Growth of yeast strains expressing recombinant sugar transporters on different carbon sources

We used the *S. cerevisiae* strain AFY-09 to screen for sugar transporters supporting growth on various carbon sources. This strain is derived from EBY.VW5000 and contains deletions of hexose and some maltose transporters to block uptake of hexoses (Wieczorke *et al.* 1999). In this strain, we integrated the gene of the *N. crassa* β-glucosidase GH1–1 under control of the *S. cerevisiae* constitutive *PGK1* promotor into the genome. We constructed high-copy expression vectors for all selected transporters, using codon optimized DNA sequences, and the strong *PGK1* promotor and *ENO1* terminator. Successful transformants were grown into the exponential phase on selective media containing 20 g/L glycerol and ethanol. We plated 5-fold serial dilutions of these cultures on agar plates containing different carbon sources and screened for growth. The plates were incubated at 30 °C and pictures of the colonies were taken after 72 hours (Fig. 2).

None of the transporter expressing strains showed a negative growth phenotype when grown on ethanol plus glycerol, indicating that the cells are not burdened by the high expression of transporters used for screening purposes (Fig. 2A). The strain background with the sugar transporter deletions that we have used in this screen is known to allow still a slight growth on hexoses, due to deletion of the glucose sensors *SNF3* and *RGT2* (Wieczorke *et al.* 1999). This explains the low background growth of the negative control strain YSS1 on some of these carbon sources. However, the spotting assay allowed us to distinguish some clear growth phenotypes on the different sugars. Compared to the parental YSS1 strain, CdtG showed clear growth on glucose, cellobiose, and lactose, with a minor growth advantage on galactose (Fig. 2B–E). Li and colleagues only tested the CdtG transporter for growth on cellobiose (Li *et al.* 2013); these results however indicate that the transporter's sugar specificity is broader than that. Interestingly, the transporter CltA did not show growth distinguishable from the parental YSS1 strain, even though in an earlier study showed it to be efficient at cellobiose uptake (Dos Reis *et al.* 2016). From the *A. niger* transporters, only expression of An03g05320 showed a clear increased growth on glucose, and indications for improved growth on cellobiose (Fig. 2B, C). However, the other four *A. niger* transporters showed only some possible small increases compared to the parental strain. Finally, the *L. starkeyi* gene supported growth on glucose and cellobiose, with some indications that it might also transport lactose and galactose (Fig. 2B–E). All strains grew mostly indistinguishable compared to the parental strain on the carbon sources mannose, fructose, sucrose, maltose and maltotriose (Fig. 2F–J).

Characterizing growth in liquid media

To get a more clear idea of how the different transporters allowed the yeast to grow on different carbon sources we conducted a second growth study, this time in liquid media using a BioScreen C MBR equipment. Exponentially growing cells expressing the different transporters were inoculated into the 100-wells plates filled with 200 µL SC-Ura-His media, containing either glycerol and ethanol, glucose, cellobiose, or lactose as a carbon source. The cells were grown at 30 °C with continuous shaking and OD_{600} was measured every 30 minutes for 116 hours. We used the growth data to calculate the average maximum growth rates and highest ODs using the R-package grofit (Fig. 3 and Table 2) (Kahm *et al.* 2010). The average growth curves of three replicates for all these cultivations is shown in Supplemental Fig. S1.

Even though the parental strain YSS1 has deletions for over 20 sugar transporters, it was also reported to have still some background growth on some hexoses due to the deletion of the

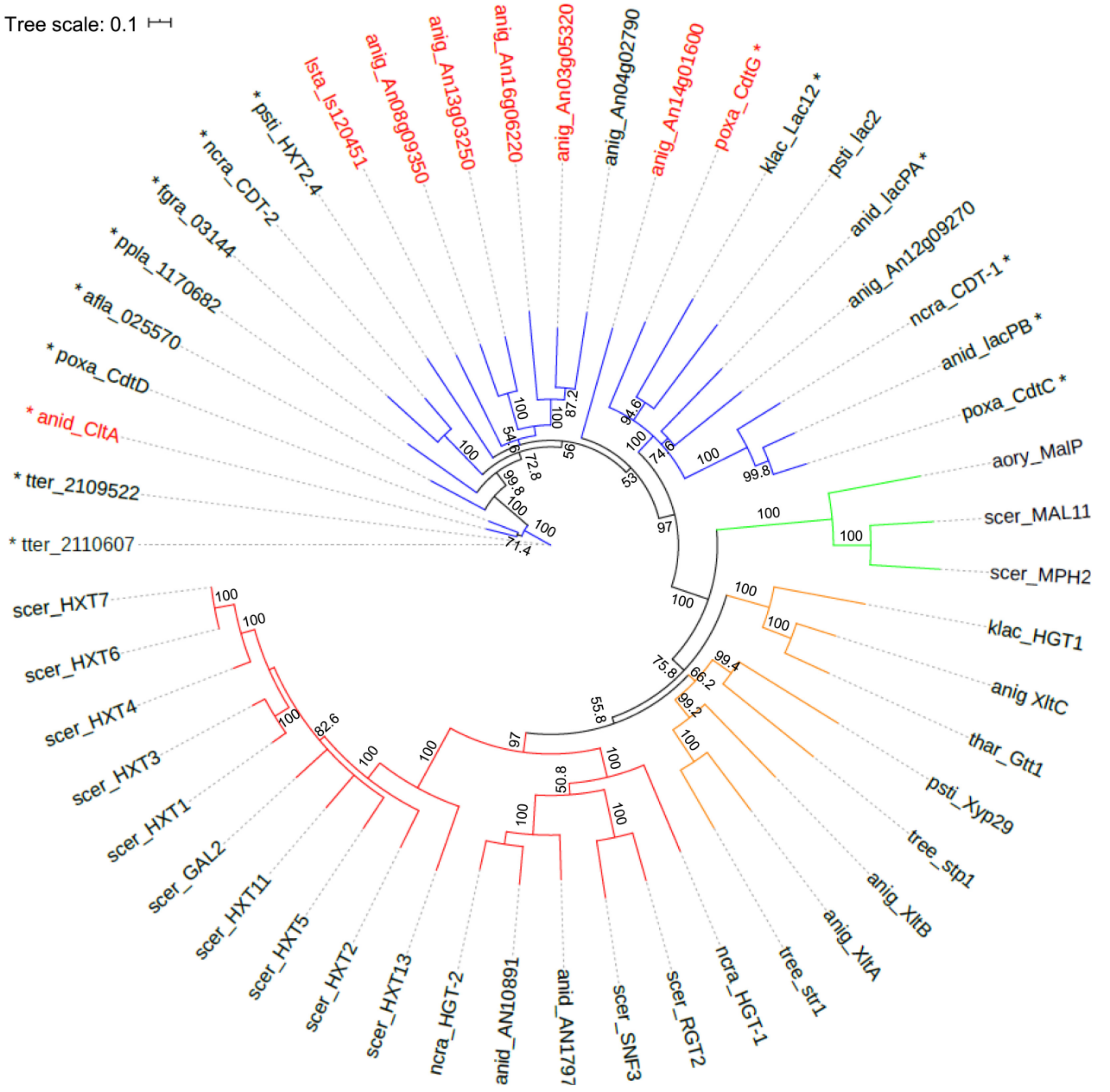

Figure 1. Phylogenetic classification of (putative) sugar transporters from various yeast and fungal hosts. The phylogenetic tree contains 51 transporter sequences in total; the names of the eight transporters characterized in this study are marked in red. (Putative) main sugar specificities of the transporters are marked by the colour of the branches, with red for glucose, orange for xylose, blue for cellobiose, longer cellodextrins, and lactose, and green for maltose and maltotriose. Transporters with a reported ability to transport cellobiose have been marked with ∗. Numbers at the nodes represent percentage bootstrap values based on 500 samplings. The abbreviation of each species is added in front of the name of the transporter genes: aory = *Aspergillus* oryzae, afla = *Aspergillus flavus*, anid = *Aspergillus nidulans*, anig = *Aspergillus niger*, fgra = *Fusarium graminearum*, klac = *Kluyveromyces lactis*, lsta = *Lipomyces starkeyi*, ncra = *Neurospora crassa*, poxa = *Penicillium oxalicum*, ppla = *Postia placenta*, psti = *Pichia stipitis*, scer = *Saccharomyces cerevisiae*, thar = *Trichoderma harzianum*, *tree* = *Trichoderma reesei* and tter = *Thielavia terrestris*.

glucose sensor genes *SNF3* and *RGT2* (Wieczorke *et al.* 1999). In these experiments, this showed by the background growth of this strain on glucose and cellobiose to an average final OD_{600} of 0.374 ± 0.014 and 0.449 ± 0.020, respectively. However, as this only happened over the course of 96 hours, the use of this parental strain still allowed the distinguishing of positive growth phenotypes caused by the expressed transporters.

Within the growth phenotypes of the full set of strains and carbon sources, lactose was an outlier as a carbon source, showing often a nearly full growth inhibition (Supplemental Fig. S1 and 2, final column). Remarkably, this negative effect also happened a few times in strains that express a transporter that does transport lactose, indicating a possible lactose toxicity. Literature suggests that this is caused by the toxic effect of lactose accumulation in the cytosol (Lodi and Donnini 2005). In our system, we use a β-glucosidase to metabolise the lactose, as previously its affinity has been shown to be sufficient for lactose fermentation (Liu *et al.* 2016). However, the 8-fold lower β-

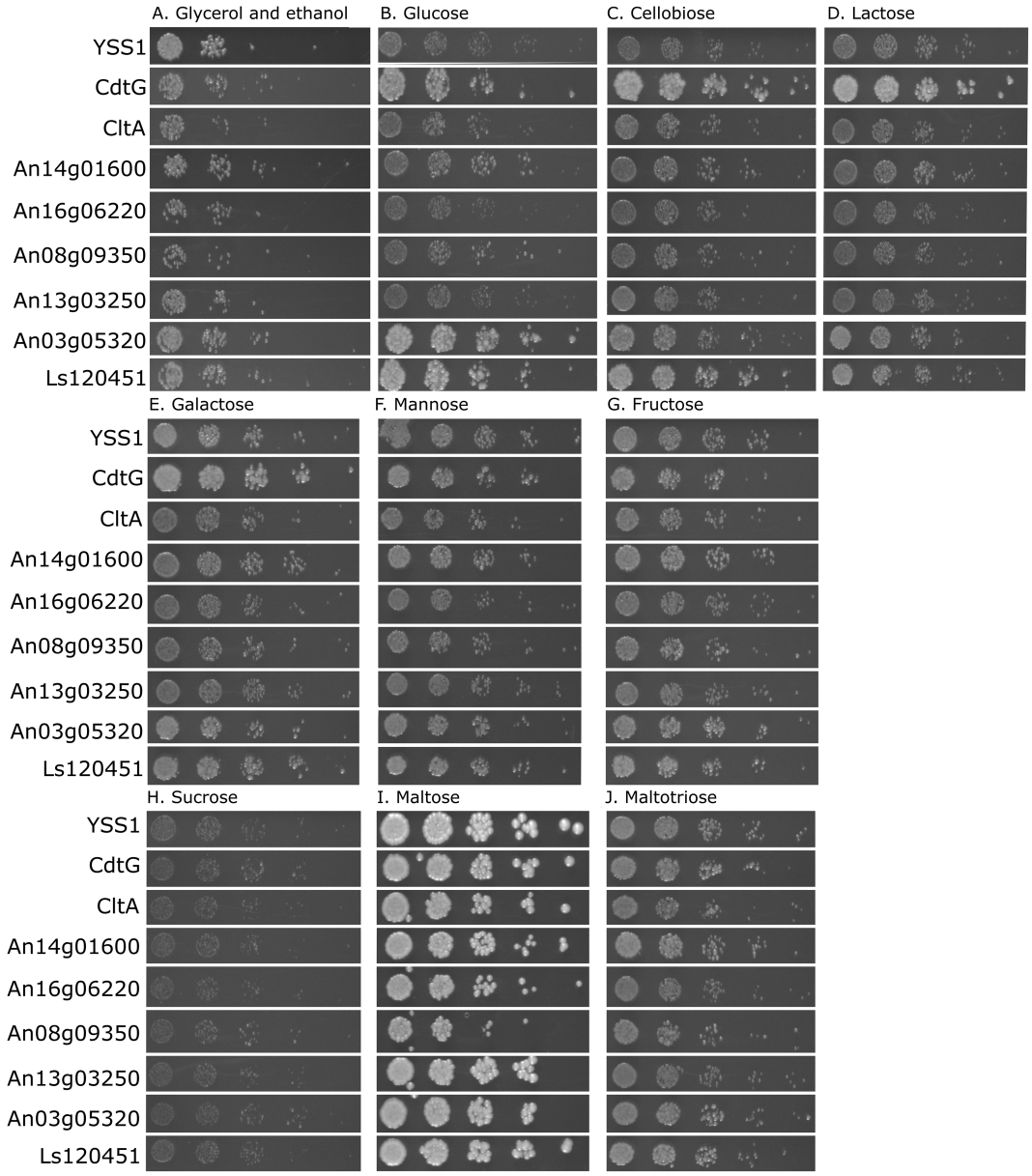

Figure 2. Expression of different sugar transporters lead to varying growth phenotypes in *S. cerevisiae*. Strains expressing the various putative sugar transporters and *N. crassa* β-glucosidase GH1–1 were grown into the exponential phases in medium containing 2% glycerol and 2% ethanol. A serial dilution was made (5-fold, starting at an OD_{600} of 0.1) and cells were spotted on agar plates containing SC-Ura-His with 2% of the carbon sources glycerol and ethanol (A), glucose (B), cellobiose (C), lactose (D), galactose (E), mannose (F), fructose (G), sucrose (H), maltose (I), and maltotriose (J).

galactosidase activity of GH1–1 could mean a prolonged lactose accumulation in the cytosol leading to cellular toxicity. In general, this made it for most strains difficult to analyse growth on lactose. This comes from the point that they did show more growth than the YSS1 controls strain, but this growth was still comparable or less than the YSS1 background growth on glucose and cellobiose.

Just as in the solid media growth analysis (Fig. 2), also in liquid growth all strains were not burdened by the expression of the transporters, as all expression strains grew comparable to the parental strain YSS1 on media containing glycerol and ethanol as carbon sources (Fig. 3A).

As reported before (Li *et al.* 2013), the strain expressing CdtG supported growth on cellobiose (Fig. 4). Here with a maximum growth rate of 0.192/h, the highest of all strains analysed in this set (Fig. 3C). However, in this study we did not observe the prolonged lag period that Li *et al.* reported. This change might be caused by the use of different promoters (pPGK1 vs pGPD1) and culture conditions (selective media vs rich media) in this and their study, respectively. In the report by Li *et* al only growth on cellobiose was analysed, but here the CdtG expressing strain also showed growth on glucose and lactose. For the latter we observed a maximum growth rate of 0.252/h, which was faster than on cellobiose and it reached a slightly higher maximum OD_{600}. The ability of CdtG to transport lactose was already hypothesized by Li *et al.* (Li *et al.* 2013). This was expected as in phylogenetic analysis CdtG clusters together with the *K. lactis* Lac12p lactose permease and the *N. crassa* CDT-1 transporter. Both of these have been shown to also be able to transport cellobiose and lactose (Sadie *et al.* 2011; Liu *et al.* 2016). In addition,

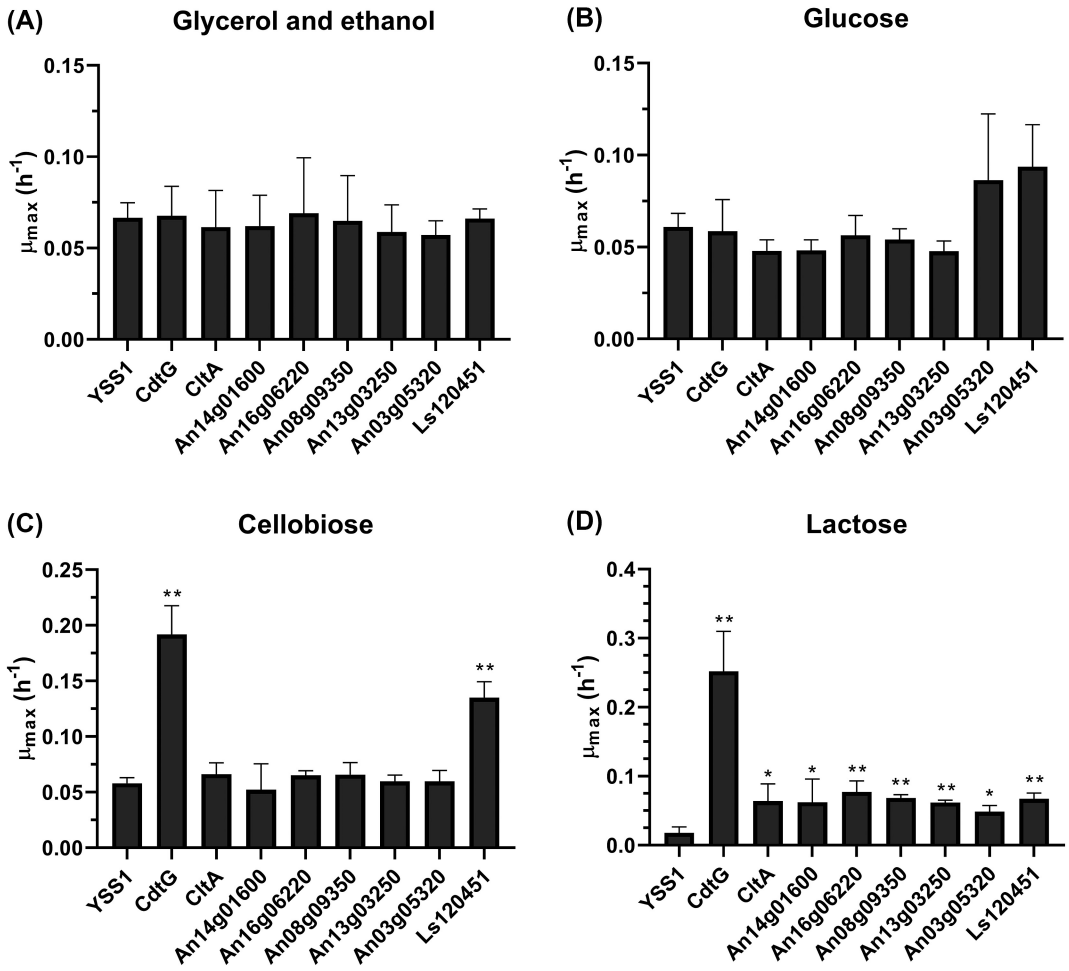

Figure 3. Maximum growth rates of strains expressing sugar transporters. Strains expressing the various putative sugar transporters and *N. crassa* β-glucosidase GH1–1 were grown on media containing the carbon sources glycerol and ethanol, glucose, cellobiose, or lactose. Data shown is the maximum growth rates calculated from three replicate cultivations and their standard deviations. $P < 0.05$ (*) and $P < 0.01$ (**) as compared to the parental strain YSS1 grown on the same carbon source, analysed by two-tailed Student's *t*-test.

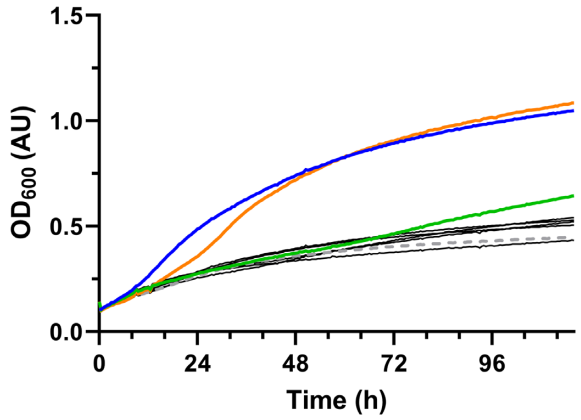

Figure 4. Growth on cellobiose of strains expressing sugar transporters. Strains expressing the various putative sugar transporters and *N. crassa* β-glucosidase GH1–1 were grown on media containing cellobiose as the carbon source. Shown are the average growth curves for three replicate cultivations. Blue = CdtG, orange = Ls120451, green = An03g05320, and the grey dashed line is the parental strain YSS1.

we showed that CdtG could also sustain growth on galactose on solid media (Fig. 2), which is also the case for Lac12p (Riley *et al.* 1987). It appears that the similarity in sequence is high enough for the proteins to have similar transporter selectivities, albeit with different sugar affinities.

In contrast to the results reported by dos Reis *et al* (Dos Reis *et al.* 2016), in our work the strain expressing CltA showed only a very small increase in growth compared to the parental strain on the different carbon sources (Fig. 3). Moreover, only when grown on cellobiose, the strain showed a very small increase in maximum growth rate, but the difference is not statistically significant and it was also much smaller than for example in CdtG and Ls120451 (Fig. 3C). However, there appear to be two different protein sequences available for the CltA gene in the online databases, and dos Reis and colleagues did not specify the sequence they got from their cDNA. This might mean that the discrepancy in results is coming from a difference in the sequence used for expression.

From the *A. niger* derived transporters, the An03g05320 strain showed most clearly growth on glucose when compared to the parental strain YSS1 (the differences in maximum growth rate were not statistically significant with $p > 0.05$) (Fig. 3B, Supplemental Fig. S1H). On cellobiose, the difference to the parental strain was small (Fig. 4), with a maximum growth rate comparable to the parental YSS1 strain (0.058 ± 0.004/h and 0.060 ± 0.005/h, for YSS1 and An03g05320, respectively).

Table 2. Maximum growth rates and maximum OD_{600} calculated from the growth curves using the R-package grofit. Data represents the average ± standard deviation of three biological replicates. $P < 0.05$ (*) and $P < 0.01$ (**) as compared to YSS1 from the same carbon source, analysed by Student's *t*-test.

	Glycerol and ethanol				Glucose				Cellobiose				Lactose			
	μ_{max}		Max OD		μ_{max}		Max OD		μ_{max}		Max OD		μ_{max}		Max OD	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
YSS1	0.067	0.007	0.568	0.037	0.061	0.006	0.374	0.014	0.058	0.004	0.449	0.020	0.018	0.007	0.292	0.097
CdtG	0.068	0.013	0.603	0.032	0.059	0.014	0.662**	0.064	0.192**	0.021	1.052**	0.106	0.252**	0.048	1.104**	0.048
CltA	0.061	0.016	0.570	0.030	0.048	0.005	0.461*	0.027	0.066	0.008	0.530	0.058	0.064*	0.021	0.546*	0.013
An14g01600	0.062	0.014	0.616	0.042	0.048	0.005	0.483**	0.029	0.052	0.019	0.543*	0.038	0.062*	0.027	0.540*	0.056
An16g06220	0.069	0.025	0.623	0.080	0.056	0.009	0.538*	0.069	0.065	0.003	0.506	0.094	0.077**	0.013	0.607*	0.024
An08g09350	0.065	0.020	0.582	0.035	0.054	0.005	0.467	0.050	0.066	0.009	0.433	0.038	0.069**	0.004	0.576*	0.005
An13g03250	0.059	0.012	0.580	0.041	0.048	0.005	0.473*	0.029	0.060	0.005	0.522*	0.030	0.062**	0.003	0.551*	0.018
An03g05320	0.057	0.006	0.606	0.042	0.086	0.029	0.839**	0.058	0.060	0.008	0.647**	0.041	0.049*	0.007	0.584*	0.038
Ls120451	0.066	0.004	0.587	0.036	0.094	0.019	0.900**	0.078	0.135**	0.012	1.087**	0.026	0.067**	0.007	0.696*	0.095

However, the parental strain YSS1 stopped growing at around 60 hours, whereas the An03g05320 expression strain showed prolonged growth. This difference became more clear after extended cultivations of up to 168 hours, where the An03g05320 strain reach a final OD about twice as high as YSS1 when grown on glucose or cellobiose ($P < 0.01$) (supplemental Fig. S2).

The other *A. niger* transporters also all showed only a little bit more growth on glucose compared to the parental strain (Supplemental Fig. S1D–G). Here as well we extended the cultivations up to 168 hours for this reason. The prolonged growth confirmed that the average final OD_{600}'s for these four strains when grown on glucose is reaching 0.6, with below 0.4 as a reference from the parental strain ($P < 0.05$ for all strains) (Supplemental Fig. S2A–D, second column). This indicates that these transporters might transport glucose into the cells, but they are not very efficient, hence the slow growth. The only strain that also showed slight growth on cellobiose after prolonged incubation was the strain expressing An13g03250. It reached higher ODs than the parental strain after 120 hours ($P < 0.05$) (Supplemental Fig. S2D). These results indicated that from the five putative cellodextrin transporters, all of them appeared to be low affinity glucose transporters, and both An13g03250 and An03G05320 were transporting cellobiose with low affinity as well.

Finally, the strain expressing the Ls120451 transporter from *L. starkeyi* grew on all three sugar substrates, with the highest growth rate (0.135 h^{-1}) and final OD_{600} (1.087) on cellobiose (Fig. 3 and 4, Table 2). The growth on glucose was second highest of this set of transporters with a μ_{max} of 0.094/h, and rather slow growth was seen on lactose. The yeast *L. starkeyi* has already been extensively used for the production of lipids, and has shown to be a versatile production host that can grow on many feedstocks (Sutanto *et al.* 2018). Recently, genetic tools have been developed to turn this yeast into a novel biotechnological host, allowing genetic modifications and expression of recombinant proteins (McNeil and Stuart 2018; Xu *et al.* 2018). However, to our knowledge no sugar transporters have to date been characterized from *L. starkeyi*, making Ls120451 the first sugar transporter of this organism described in literature.

Further characterization of transport properties of CdtG and Ls120451

The results from the two growth experiments showed that the transporters CdtG and Ls120451 were the most interesting cellobiose transporters from the transporters analysed in this study. Therefore, we selected these two for further characterization of growth on longer cellodextrins, cellobiose consumption, and method of cellobiose transport.

Previously, it was shown that in *S. cerevisiae* the combination of expression of CDT-1 and GH1–1 can sustain growth on the longer cellodextrins cellotriose and cellotetraose (Galazka *et al.* 2010). We wanted to see if this was also the case for the transporters CdtG and Ls120451. Therefore, strains expressing CdtG or Ls120451 were grown into exponential phase and used to inoculate fresh 100 μL cultivations in Eppendorf tubes containing 20 g/L cellobiose, 20 g/L cellotriose, or 20 g/L cellotetraose as the carbon source. The strains were incubated at 30 °C at 800 rpm and growth was monitored for up to 8 days using a cell counter. As shown above, both strains grew using cellobiose as the carbon source. However, neither of the strains grew on cellotriose, nor on cellotetraose (Supplemental Fig. S3).

We studied the cellobiose consumption of these two transporter expression strains by incubating exponentially growing

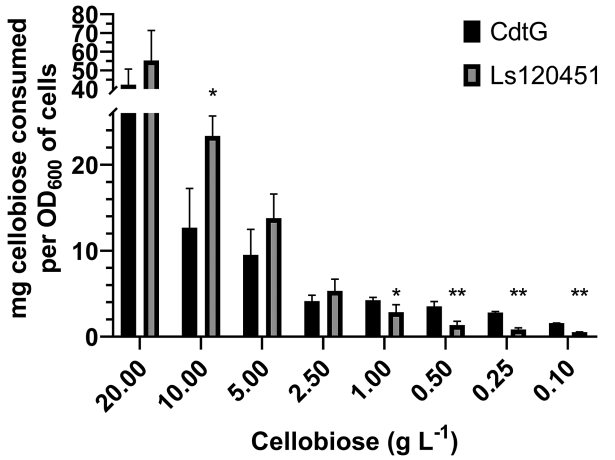

Figure 5. Cellobiose consumption of strains expressing transporters CdtG and Ls120451. Exponentially growing cells were concentrated and incubated with various concentrations of cellobiose, ranging from 0.10 to 20 g/L. The remaining cellobiose content was measured after 30 minutes and normalized to 1 OD_{600} of cells. The data shown is the average of three replicates together with the standard deviation. $P < 0.05$ (*) and $P < 0.01$ (**) as compared to CdtG from the same amount of cellobiose, analysed by two-tailed Student's *t*-test.

cells resuspended at a high OD_{600} for 30 minutes with various concentrations of cellobiose. We measured the residual cellobiose in the supernatant with HPAEC-PAD. The amount of consumed cellobiose was normalized to one OD_{600} and is shown in Fig. 5. The data showed that CdtG consumed more cellobiose than Ls120451 at cellobiose concentrations of 1.0 g/L and below ($P < 0.05$). In contrast, Ls120451 consumed slightly more cellobiose at concentrations of 2.5 g/L or higher. However, this difference was only statistically significant for 10 g/L cellobiose (Fig. 5).

Finally, we wanted to gain some insight in the transport mechanism for cellobiose for these two transporters. We tested both strains for possible cellobiose/proton symporter activity using a pH based assay that was described before (Du, Li and Zhao 2010). Exponentially growing cells were adjusted to pH 5 before the addition of a pulse of cellobiose (20 g/L final concentration) and we measured the pH response afterwards (Fig. 6A–C). For the strain expressing CdtG, this led to a small initial increase in the pH of the cell suspension, which was absent in both the LS120451 strain and the negative control YSS1 ($P < 0.05$). After this short initial alkalization, the metabolism starts to decrease the pH (Fig. 6A). In the strain expressing Ls120451 (Fig. 6B), this first initial response was not present and the pH starts to decrease after the cellobiose pulse. For the negative control YSS1 (Fig. 6C), there was no change in pH after the addition of the cellobiose, neither upwards, nor downwards. These results indicated that CdtG is possibly having sugar/proton symporter characteristics for cellobiose, while Ls120451 might be a facilitator. In order to learn more about the transport mechanism we also conducted the same experiment after incubating the cells with 25 μM carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (Fig. 6E–F). This compound uncouples the proton gradient over the plasma membrane by increasing its proton permeability. Sensitivity of cellobiose transport to this compound would indicate that the transporter uses the plasma membrane proton gradient for transport, like for example CDT-1 (Kim *et al.* 2014). Strains with the CdtG transporter (Fig. 6E) showed a loss of both the alkalization and the acidification part of the response to the cellobiose pulse when compared to the curve without CCCP ($P < 0.05$). The curve became similar to the negative control YSS1 (Fig. 6F), indicating a loss of cellobiose transport when the proton gradient is disrupted. For strains with the Ls120451 transporter, the shape of the pH signal response remained similar after CCCP incubation, indicating that the transporter does not utilize the plasma membrane protein gradient (Fig. 6E). For the negative control YSS1, there was again no change in pH signal after addition of cellobiose (Fig. 6F)

Shake flask growth of strains expressing CdtG and Ls120451

We characterized the growth patterns on cellobiose of the CdtG and Ls120451 strains a bit more in-depth in a larger scale. We grew both strains in 250 mL shake flasks in 50 mL of media containing 20 g/L cellobiose as carbon source. We measured OD_{600}, cellobiose consumption, and ethanol production for 96 hours (Fig. 7, Table 3). In these cultivations, we observed no lag time, which can be contributed to cellobiose also being the carbon source in the precultures, a phenomenon also observed by Bae et al (Bae *et al.* 2014). The CdtG expressing strain grew slightly faster than the strain expressing Ls120451, with calculated maximum growth rates of 0.194 ± 0.012 and 0.142 ± 0.006, respectively ($p > 0.05$). In the end of the cultivation, they reached similar final OD_{600} values after 96 hours (Table 3). For the first 24 hours the cellobiose consumption profiles were similar and both strains consumed around 8.5 g/L cellobiose, after this the cellobiose consumption of Ls120451 continued at a higher rate than CdtG, reaching a final cellobiose concentration of 7.2 and 8.9 g/L, respectively. The Ls120451 expression strain also had a 33% higher final ethanol concentration and a 23% higher ethanol yield than the CdtG strain ($P < 0.01$) (Table 3).

These results showed the two transporters to be moderate cellobiose transporters compared to some of the results reported in literature. Galazka *et al* reported in their first CDT-1 paper an ethanol yield of 0.441 ± 0.001 grams ethanol per gram of cellobiose (Galazka *et al.* 2010), while the two transporters studied here reach only 0.14 ± 0.05 and 0.18 ± 0.01 g/g, for CdtG and Ls120451, respectively. However, it has to be kept in mind that we have used a hexose transporter deficient strain with a single copy integration of the GH1–1 gene. It has been shown that increasing the copy number of GH1–1 can improve cellobiose fermentation significantly (Oh *et al.* 2016; Kim *et al.* 2019). Bae et al reported a set of five transporters from *P. chrysogenum, T. reesei*, and *T. terrestris,* which showed ethanol yields ranging from 0.07 ± 0.01 up to 0.31 ± 0.01, which puts the two transporters reported here in the middle of their set (Bae *et al.* 2014). Even though these transporters are currently only showing moderate transport characteristics, it should be kept in mind that the cellobiose transport of these transporters could still be improved through protein engineering. The most promising approach so far for this has been through directed evolution studies, which have for example already significantly improved transport characteristics and increased cellobiose fermentation for HXT2.4 (Ha *et al.* 2013b), CDT-1 (Eriksen *et al.* 2013), and CDT-2 (Lian *et al.* 2014).

Looking at the results from the cellobiose consumption assay, it is unexpected that a relatively large amount of cellobiose was left in the cultures even after 96 hours. After all, both strains showed to be able to transport cellobiose even at concentrations as low as 0.25 g/L cellobiose (Fig. 5). This is much lower than the measured residual concentrations of 7.2 and 8.9 g/L.

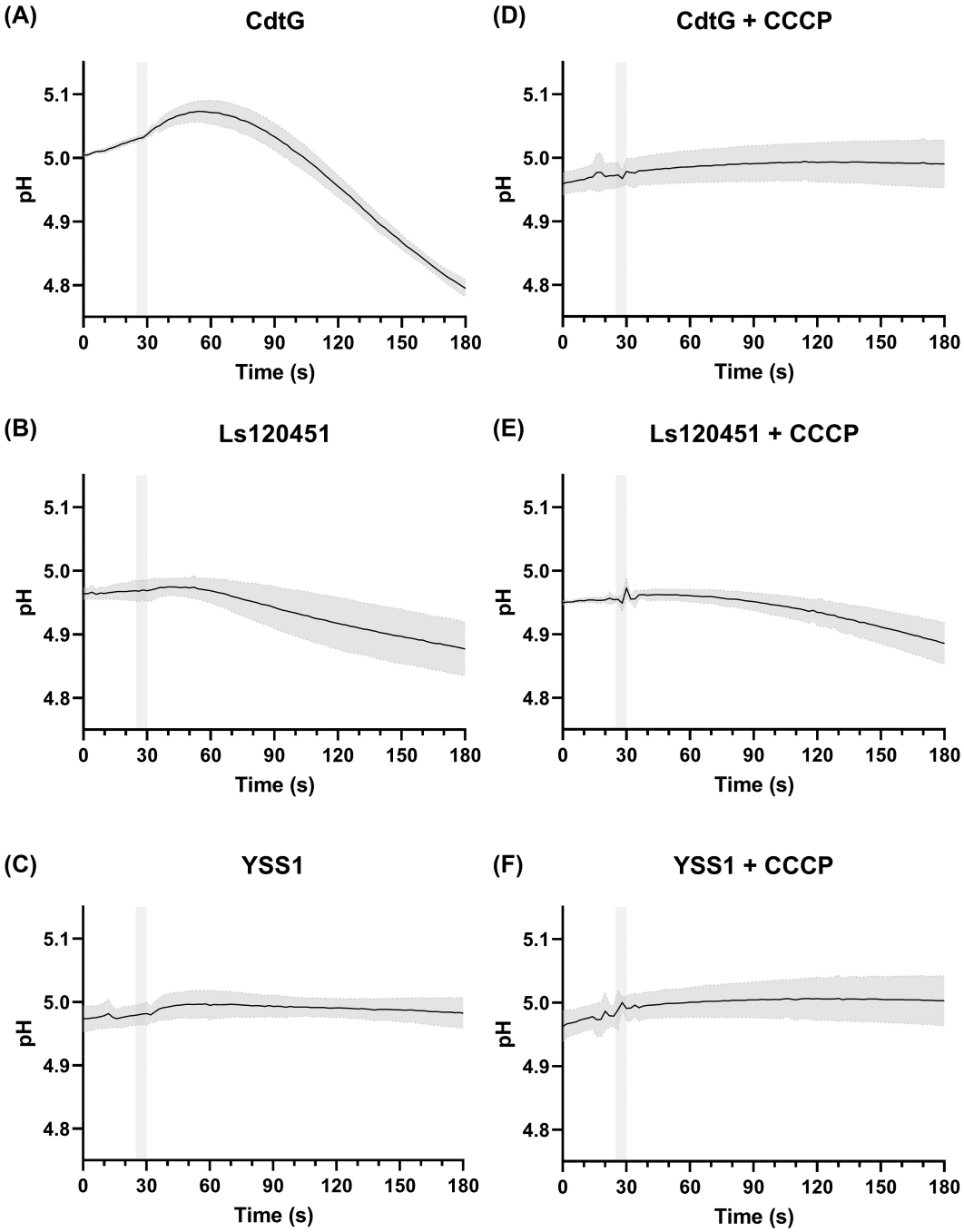

Figure 6. CdtG shows possible sugar/proton symporter characteristics and Ls120451 does not. Exponentially growing cells expressing CdtG (A), Ls120451 (B), and the negative control YSS1 (C) were adjusted to pH 5, followed by addition of cellobiose to a 20 g/L final concentration (marked with light grey bar). The experiment was repeated with a pre-incubation with 25 μM CCCP for CdtG (D), Ls120451 (E), and the parental strain YSS1 as negative control (F). The data shown is the average from three replicates with the standard deviation shaded in grey.

However, the efficiency of cellobiose uptake might be influenced by the acidification of the media by the yeast cells during growth. A sensitivity to low pH has also been found for the cellobiose transporters CDT-1 and CDT-2 from *N. crassa* (Turner *et al.* 2016; Oh *et al.* 2017). In general, if this would be the problem for these transporters it could be possible to optimize this through protein engineering, just as has been done succesfully for CDT-2 (Oh *et al.* 2017).

In addition to the cellobiose measured in the extracellular medium, we also detected peaks in the HPLC spectra that indicated the appearance of small amounts of glucose in the culture supernatant. We hypothesized that this might result from secreted β-glucosidase cleaving the cellobiose outside the cells. However, we measured the extracellular β-glucosidase activities and found them to be negligible (data not shown), indicating that the source of the glucose is most probably glucose efflux

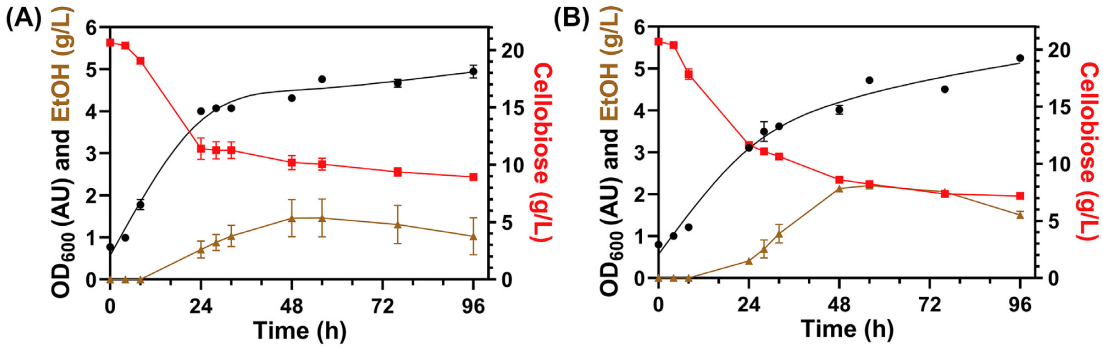

Figure 7. Cellobiose fermentation of CdtG and Ls120451 expressing strains in shake flask scale. Strains expressing CdtG (A) or Ls120451 (B) were grown in 50 mL medium with 20 g/L cellobiose in shake flasks. OD_{600} (black, •), cellobiose (red, ■) and ethanol (brown, ▲) were monitored over 96 hours to asses differences in growth characteristics. The data shown is the average of duplicate cultures with error bars depicting the range.

Table 3. Cellobiose growth characteristics for strains expressing CdtG and Ls120451.

Strain	Maximum growth rate (h^{-1})	OD_{600}[a]	Cellobiose consumption (g/L/h)[b]	Final ethanol concentration (g/L)[a]	Ethanol yield (g/g cellobiose)[a]	Productivity (g/L/h)[a]
CdtG	0.194 ± 0.012	4.77 ± 0.05	0.19 ± 0.01	1.46 ± 0.45	0.14 ± 0.05	0.03 ± 0.01
Ls120451	0.142 ± 0.006	4.72 ± 0.03	0.20 ± 0.01	2.20 ± 0.06	0.18 ± 0.01	0.05 ± 0.00

Each value is the average of two replicates with the range of measurements.
[a]Values for OD_{600}, final ethanol concentration, ethanol yield and productivity were calculated from 56 h time point.
[b]Cellobiose consumption rates were calculated from 8 to 56 hours.

from the cells. Previous studies employing cellodextrin transporters and an intracellular β-glucosidase have reported similar results for the export of glucose and longer cellodextrins (Ha *et al.* 2011, 2013a; Bae *et al.* 2014). It was hypothesized that an imbalance between the expression level of the transporter and the β-glucosidase is the cause of this efflux, as a lower transporter expression level decreased glucose efflux levels (Ha *et al.* 2011).

CONCLUSIONS

In this study, we screened a set of eight (putative) sugar transporters from various yeast and fungal origins on solid and in liquid media for their growth on various carbon sources. From these, the transporters CdtG from *P. oxalicum* and Ls120451 from *L. starkeyi* showed interesting cellobiose transport characteristics for growth in *S. cerevisiae*. In addition to cellobiose, CdtG also showed growth on glucose, lactose, and galactose. Ls120451 also showed growth on glucose and slow growth on lactose. However, compared to previously described cellobiose transporters like CDT-1 and CDT-2, in our hexose transporter null mutant expression strain these two transporters only showed moderate fermentation capabilities and they might need engineering to improve this. The transporter Ls120451 is the first sugar transporter characterized from the yeast *L. starkeyi* to date.

MATERIALS AND METHODS

Plasmids and strain constructions

All DNA sequences encoding the transporters were *S. cerevisiae* codon optimized and synthesized as gene blocks by Thermofisher Scientific (Espoo, Finland), genes sequences were flanked by BsaI and BsmBI restriction sites for use with the yeast MoClo based toolkit (Lee *et al.* 2015). After insertion of the genes into entry vectors all expression vectors were constructed using Gibson assembly. The constructs made (Table 4) were similar, having a ColE1 *Escherichia coli* origin and Kanamycin selection marker, and a 2-μ yeast origin of replication with a *URA3* selection marker. The transporter genes were put under the control of the *S. cerevisiae PGK1* promoter and *ENO1* terminator. Plasmids were amplified in DH5α *E. coli* strains and correct assembly of the plasmids was confirmed with sequencing.

Table 4. Plasmids used in this study.

Name	Relevant characteristics	Reference
pJR030a	2 μm, *KanR*, *URA3*, *pPGK1-CdtG-tENO1*	This study
pJR032a	2 μm, *KanR*, *URA3*, *pPGK1-CltA-tENO1*	This study
pJR034a	2 μm, *KanR*, *URA3*, *pPGK1-An14g01600-tENO1*	This study
pJR035a	2 μm, *KanR*, *URA3*, *pPGK1-An16g06220-tENO1*	This study
pJR036a	2 μm, *KanR*, *URA3*, *pPGK1-An08g09350-tENO1*	This study
pJR037a	2 μm, *KanR*, *URA3*, *pPGK1-An13g03250-tENO1*	This study
pJR038a	2 μm, *KanR*, *URA3*, *pPGK1-An03g05320-tENO1*	This study
pJR040a	2 μm, *KanR*, *URA3*, *pPGK1-Ls120451-tENO1*	This study

Transporters were screened in the *S. cerevisiae* hexose transporter deficient strain YAF-09, which is EBY.VW5000 (Wieczorke *et al.* 1999) with additional deletion of *MAL13* and *MAL21*. The gene encoding the *N. crassa* β-glucosidase GH1–1 was put under control of the *PGK1* promoter and integrated into the *HIS2* locus, creating strain YSS1. The transporter expression plasmids were transformed into the YSS1 parental strain using the LiAc-method (Gietz *et al.* 1992). Proper transformants (Table 5) were

Table 5. Yeast strains used in this study.

Strain	Genotype	Reference
EBY.VW5000	*hxt1–16Δ gal2Δ stl1Δ agt1Δ ydl247wΔ yjr160cΔ snf3Δ rgt2Δ*	Wieczorket *et al.* (1999)
YSS1	AFY-09: *his3Δ::pPGK1-ncGH1–1-tENO1*	This study
YJR248	YSS1 + pJR030a(*URA3, pPGK1-CdtG-tENO1*)	This study
YJR250	YSS1 + pJR032a(*URA3, pPGK1-CltA-tENO1*)	This study
YJR251	YSS1 + pJR034a(*URA3, pPGK1-An14g01600-tENO1*)	This study
YJR252	YSS1 + pJR035a(*URA3, pPGK1-An16g06220-tENO1*)	This study
YJR253	YSS1 + pJR036a(*URA3, pPGK1-An08g09350-tENO1*)	This study
YJR254	YSS1 + pJR037a(*URA3, pPGK1-An13g03250-tENO1*)	This study
YJR255	YSS1 + pJR038a(*URA3, pPGK1-An03g05320-tENO1*)	This study
YJR256	YSS1 + pJR040a(*URA3, pPGK1-Ls120451-tENO1*)	This study

selected on synthetic medium plates supplemented with the appropriate auxotrophic amino acid mixture.

Cultivation conditions

The yeast strains were routinely cultivated at 30 °C in either rich medium (YP, 10 g/L yeast extract, 20 g/L peptone), or in synthetic complete (SC) medium (6.7 g/L yeast nitrogen base supplemented with the appropriate amino acid mixture), for selection based on auxotrophy. Carbon sources were either glucose, cellobiose, or lactose at 20 g/L, or both glycerol and ethanol at 20 g/L each.

Analysis of growth on various carbon sources

The yeast strains expressing the transporters were grown into exponential fase in 24-well plates. Cells were collected and washed twice with ddH_2O, followed by resuspension in ddH_2O at a stock concentration of 2 OD_{600}/mL. For growth on solid agar media, the cell suspensions were diluted to an OD_{600} of 0.1, followed by 5-fold serial dilutions made in a 96-well plate format. A replica plater was used to transfer 2 μL of the suspension to square agar plates containing SC-Ura stock with 20 g/L of the following carbon sources: glucose, galactose, fructose, mannose, xylose, cellobiose, lactose, sucrose, maltose, maltotriose, or glycerol and ethanol.

For screening analysis of growth in liquid media, 20 μL of the 2 OD_{600}/mL yeast stock-solution was used to inoculate the wells of a honeycomb 100-well plate containing 200 μL of SC-Ura stock supplemented with either glucose, cellobiose, lactose, or both glycerol and ethanol at 20 g/L each. The plate was incubated in a Bioscreen C MBR system (Oy Growth Curves Ab Ltd, Helsinki, Finland), which was set at 30 °C, maximum shaking with normal amplitude. The OD_{600} was measured every thirty minutes for 116 or 168 hours.

Small-scale cultivations were used to test for growth on cellotriose and cellotetraose. 500 μL Eppendorf tubes were filled with 100 μL of SC-Ura medium containing 20 g/L of cellobiose, cellotriose, or cellotetraose. Cultivations were grown at 30 °C, with 800 rpm shaking. Cell amounts were counted using a LUNA-II automated cell counter (Logos Biosystems, Anyang, Republic of South Korea) for up to 8 days.

In larger scale cultivations, 50 mL of SC-Ura medium with 20 g/L cellobiose was inoculated from an overnight preculture at a starting OD_{600} of 0.8. The shake flask cultivations were performed at 30 °C and 230 rpm for 96 hours. Samples were taken for OD_{600} measurements and analysis of cellobiose, ethanol and glucose concentrations using HPLC. Compounds were separated with Fast Acid Analysis Column (100 × 7.8 mm, BioRad Laboratories, Helsinki, Finland) and Aminex HPX-87H organic acid analysis column (300 × 7.8 mm, BioRad Laboratories, Helsinki, Finland) connected to Waters 2690 separation module. Peaks were detected with Waters 2414 differential refractometer (Waters, Milford, MA). The column was eluted with 5 mM H_2SO_4 at a 0.5 mL/min flow rate at 55 C.

Cell based sugar consumption assay

The cell based cellobiose consumption assay was adapted from the paper by Zhang and colleagues (Zhang *et al.* 2017). In short, the transporter expression strains were grown into the exponential phase and harvested at an OD_{600} 1–2. The pellets were washed twice with assay buffer (100 mM KPO_4 Buffer, 100 mM NaCl, pH 6.5) and resuspended to 60 OD_{600}/mL. Cellobiose stocks were prepared in assay buffer ranging from 40 to 0.2 g/L. 500 μL of cell suspension was mixed with 500 μL of cellobiose stock and incubated for 30 minutes in a heater-shaker at 30 °C and 1000 rpm. Samples were centrifuged for 5 minutes at 14 000 rcf, 0 °C and supernatant was removed for analysis using HPAEC-PAD as described before (Tenkanen *et al.* 1997).

Symporter assay

Transporter cellobiose/proton symporter activity was assessed as described before (Du, Li and Zhao 2010). Exponentially growing cells were harvested at an $OD_{600} \approx 1$ and washed twice with ice-cold water before storing 1 mL aliquots at 100 OD_{600}/mL on ice. Five minutes before the assay, 1 mL of cells was adjusted to water in a final volume of 16 mL and incubated at 30 °C. Next, under continuous stirring, we adjusted this unbuffered cell suspension to pH 5.0 and used a Mettler Toledo T70 titrator (Mettler Toledo, Columbus, OH) to measure the pH change caused by an addition of a pulse of 20 g/L cellobiose (final concentration). If the pH increases after addition of the cellobiose, a proton/sugar symporter behaviour of the transporter is suggested. We added 1 μL of a 1000X stock solution of carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (final concentration 25 μM) to the cells 5 minutes before the assay to investigate the dependency of the change in pH on the proton gradient over the plasma membrane.

Bioinformatics methods

The BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for sequence similarity searches. The Geneious software (version 8.1.9, http://www.geneious.com) was used for the

multiple sequence alignments and phylogenetic tree construction of Fig. 1. First, all 51 protein sequences were aligned using the CLustalW alignment function with default parameters. The aligned sequences were then used as the basis for the phylogenetic tree construction using the Geneious Tree Builder function. For this, we used the Jukes-Cantor genetic distance model, Neighbor-Joining tree build method and 500 bootstraps. The resulting tree was visualized using iTOL (Letunic and Bork 2019). Maximum growth rates were calculated from the growth curves using the R-package grofit (Kahm *et al.* 2010).

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

AUTHORS' CONTRIBUTIONS

JR, KI, and MP designed the experiments. JR conducted all the laboratory work and prepared the manuscript. JR, KI, and MP revised and improved the manuscript. All authors have read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable for this study.

CONSENT FOR PUBLICATION

Not applicable for this study.

Conflicts of Interests

The authors declare that they have no competing interests.

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