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*.

Editor: John Morrissey

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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 (Aditya

Received: 16 December 2019; Accepted: 17 April 2020

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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 cellobextrins that are released by the cellulases and it requires additional \(\beta\)-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 cellobextrins. In 2010, it has been reported that a minimal system for cellobextrin fermentation by S. cerevisiae consists of recombinant expression of a cellobextrin transporter, together with an intracellular \(\beta\)-glucosidase to hydrolyse the cellobextrins (Galazka et al. 2010). Galazka et al. introduced the Neurospora crassa cellobextrin transporters CDT-1 or CDT-2 into S. cerevisiae and combined their expression with the intracellular expression of the \(\beta\)-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 metabolize 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 simultaneously, 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 \(\beta\)-glucosidase from a different host (Bae et al. 2014), or replaced by a phoshorylitic enzyme, for example a cellobextrin 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 cellobextrin 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 cellobextrins 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 \(\beta\)-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 cellobextrin 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 \(\beta\)-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 cellobextrins (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 cellobextrin transporters which phylogenetically clustered together with known cellobextrin and lactose transporters (including both CDT-1 and CDT-2) (Peng et al. 2018). From this group, we selected the genes An14g01600, An16p06220, 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 cellobextrin transporter by dos Reis et al (Dos Reis et al. 2016). The latter gene is part of a family of cellobextrin 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
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, celldextrins, 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).

### 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 |
|                       | An03g05520       | XP_0 013 90413.1           | Peng et al. 2018 |
| Aspergillus nidulans  | CdtG             | AGN74922.1                 | Li et al. 2013  |
|                       | CltA             | XP_681 616.1               | dos Reis et al. 2016 |

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 OD600 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 (Kahn 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 cellohextans, 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, tre = 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 OD600 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).
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 OD600 calculated from the growth curves using the R-package grofit. Data represents the average ± standard deviation of three biological replicates.

|          | Glucose | Glycerol | Ethanol | Lactose |
|----------|---------|----------|---------|---------|
| **μmax** | **μ**06 | **μ**06  | **μ**06  | **μ**06  |
| **YSS1** | 0.067   | 0.067   | 0.067   | 0.067   |
| **CdtG** | 0.068   | 0.068   | 0.068   | 0.068   |
| **Ls120451** | 0.067 | 0.067 | 0.067 | 0.067 |

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. S2). These results indicated that from the five putative celldextrin 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 (MC Neil 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 celldextrins, 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 celldextrins celluliose 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 mL cultivations in Eyppendorf 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...
cells resuspended at a high OD_{600} for 30 minutes with various concentrations of cellubiose. We measured the residual cellubiose in the supernatant with HPAEC-PAD. The amount of consumed cellubiose was normalized to one OD_{600} and is shown in Fig. 5. The data showed that CdtG consumed more cellubiose than Ls120451 at cellubiose concentrations of 1.0 g/L and below (P < 0.05). In contrast, Ls120451 consumed slightly more cellubiose at concentrations of 2.5 g/L or higher. However, this difference was only statistically significant for 10 g/L cellubiose (Fig. 5).

Finally, we wanted to gain some insight in the transport mechanism for cellubiose for these two transporters. We tested both strains for possible cellubiose/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 cellubiose (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 cellubiose pulse. For the negative control YSS1 (Fig. 6C), there was no change in pH after the addition of the cellubiose, neither upwards, nor downwards. These results indicated that CdtG is possibly having sugar/proton symporter characteristics for cellubiose, 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 carbonylcyanidem-chlorophenylhydrazine (CCCP) (Fig. 6D–F). This compound uncouples the proton gradient over the plasma membrane by increasing its proton permeability. Sensitivity of cellubiose 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 cellubiose 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 cellubiose 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 cellubiose (Fig. 6F).

Shake flask growth of strains expressing CdtG and Ls120451

We characterized the growth patterns on cellubiose 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 cellubiose as carbon source. We measured OD_{600}, cellubiose consumption, and ethanol production for 96 hours (Fig. 7, Table 3). In these cultivations, we observed no lag time, which can be contributed to cellubiose 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 cellubiose consumption profiles were similar and both strains consumed around 8.5 g/L cellubiose, after this the cellubiose consumption of Ls120451 continued at a higher rate than CdtG, reaching a final cellubiose 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 cellubiose 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 cellubiose (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 cellubiose 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. terestris, 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 cellubiose 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 cellubiose 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 cellubiose consumption assay, it is unexpected that a relatively large amount of cellubiose was left in the cultures even after 96 hours. After all, both strains showed to be able to transport cellubiose even at concentrations as low as 0.25 g/L cellubiose (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 successfully 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.
from the cells. Previous studies employing celloextrin transporters and an intracellular β-glucosidase have reported similar results for the export of glucose and longer celloextrins (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. oxalici um 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 Bsal 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 CoE1 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.

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 4. Plasmids used in this study.

| Name | Relevant characteristics | Reference |
|------|--------------------------|-----------|
| pJR030a | 2 μm, KanR, URA3, pPGK1-CdtG-ENO1 | This study |
| pJR032a | 2 μm, KanR, URA3, pPGK1-Clta-ENO1 | This study |
| pJR034a | 2 μm, KanR, URA3, pPGK1-An14g01600-ENO1 | This study |
| pJR035a | 2 μm, KanR, URA3, pPGK1-An16g06220-ENO1 | This study |
| pJR036a | 2 μm, KanR, URA3, pPGK1-An08g09350-ENO1 | This study |
| pJR037a | 2 μm, KanR, URA3, pPGK1-An13g09350-ENO1 | This study |
| pJR038a | 2 μm, KanR, URA3, pPGK1-An03g05320-ENO1 | This study |
| pJR040a | 2 μm, KanR, URA3, pPGK1-Ls120451-ENO1 | This study |

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. OD600 (black, ◦), cellobiose (red, ■) and ethanol (brown, ▲) were monitored over 96 hours to assess 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) | OD600a | Cellobiose consumption (g/L/h)b | Final ethanol concentration (g/L)b | 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.

aValues for OD600, final ethanol concentration, ethanol yield and productivity were calculated from 56 h time point.

bCellobiose consumption rates were calculated from 8 to 56 hours.
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 phase in 24-well plates. Cells were collected and washed twice with ddH₂O, followed by resuspension in ddH₂O at a stock concentration of 2 OD₆₀₀/mL. For growth on solid agar media, the cell suspensions were diluted to an OD₆₀₀ 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 OD₆₀₀/mL yeast-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, with maximum shaking with normal amplitude. The OD₆₀₀ was measured every thirty minutes for 116 or 168 hours.

Small-scale cultivations were used to test for growth on cellobiose and cellobiose. 500 μL Eppendorf tubes were filled with 100 μL of SC-Ura medium containing 20 g/L of cellobiose, cellobiose, or cellobiose. 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 OD₆₀₀ of 0.8. The shake flask cultivations were performed at 30 °C and 230 rpm for 96 hours. Samples were taken for OD₆₀₀ 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 H₂SO₄ 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 OD₆₀₀ 1–2. The pellets were washed twice with assay buffer (100 mM KPO₄ Buffer, 100 mM NaCl, pH 6.5) and resuspended to 60 OD₆₀₀/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 of 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 OD₆₀₀ ≈ 1 and washed twice with ice-cold water before storing 1 mL aliquots at 100 OD₆₀₀/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 T77 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 for 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.

ACKNOWLEDGMENTS
The authors would like to thank Prof. Dr. Eckhard Boles for providing the S. cerevisiae strain AFY-09. The authors would like to acknowledge Sami Havukainen and Robert Pylkkänen for technical assistance and valuable discussions.

FUNDING
This work was supported financially by the Academy of Finland through research grant SA-FOSSOK [Decision No. 309384]. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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