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Laboratory evolution and physiological analysis of *Saccharomyces cerevisiae* strains dependent on sucrose uptake via the *Phaseolus vulgaris* Suf1 transporter

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ABSTRACT

Knowledge on the genetic factors important for efficient expression of plant transporters in yeast is still very limited. Here, we investigated if laboratory evolution could improve the expression a sucrose transporter in yeast and which mutations were crucial for the evolved phenotype. *Pv*Suf1 (*Phaseolus vulgaris* sucrose facilitator 1) was key in a previously published strategy aimed at increasing ATP yield in *S. cerevisiae* since it has been described as an uniporter. However, attempts to construct yeast strains in which sucrose metabolism was dependent on *PvSUF1* led to slow sucrose uptake. Here, *PvSUF1*-dependent *S. cerevisiae* strains were evolved for faster growth. Of five independently evolved strains, two showed an approximately two-fold higher anaerobic growth rate on sucrose than the parental strain (μ = 0.19 h^{-1} and μ = 0.08 h^{-1}, respectively). All five mutants displayed sucrose-induced proton uptake (13-50 µmol H^+ (g biomass)$^{-1}$ min^{-1}). Their ATP yield from sucrose dissimilation, as estimated from biomass yields in anaerobic chemostat cultures, was the same as that of a congenic strain expressing the native sucrose symporter Mal11p. Four out of six observed amino acid substitutions encoded by evolved *PvSUF1* alleles removed or introduced a cysteine residue and may be involved in transporter folding and/or oligomerization. Expression of one of the evolved *PvSUF1* alleles (*PvSUF1*$^{I209F\ C265F\ G326C}$) in an unevolved strain enabled it to grow on sucrose at the same rate (0.19 h^{-1}) as the corresponding evolved strain. This study shows how laboratory evolution can be used to improve sucrose uptake via heterologous plant transporters; sheds light into the importance of cysteine residues for efficient plant transporter expression in yeast and warrant reinvestigation of *Pv*Suf1's mechanism in a plant context.

KEY WORDS: plant transporter expression, laboratory evolution, yeast physiology, plant sucrose facilitator, sucrose uptake.

INTRODUCTION

Sucrose (α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside) is an intensively used carbon source in microbial biotechnology (Maiorella, Blanch, & Wilke, 1984; Marques, Raghavendran, Stambuk, & Gombert, 2016; Peters, Rose, & Moser, 2010) and plays a vital role as a carbon and energy carrier in plants (Salerno & Curatti, 2003). In plants, *s*ucrose exits source cells via plasmodesmata as well as via membrane transport mediated by sucrose uniporters (Doidy et al., 2012).

In *Saccharomyces cerevisiae*, a yeast employed in many different industrial bioprocesses, the predominant pathway for sucrose catabolism starts with its extracellular hydrolysis, catalysed by invertase. The free energy available from sucrose hydrolysis ($\Delta G_{0'}$ = -29 kJ/mol)(Goldberg, Tewari, & Ahluwalia, 1989) is not conserved by the yeast cells. The resulting monomers glucose and fructose are subsequently transported into the cells by facilitated diffusion. Alternatively, after its uptake via proton symporters, sucrose can also be hydrolysed in the cytosol (Marques et al., 2017; Stambuk, Silva, Panek, & Araujo, 1999).

Inspired by previous studies on free-energy conservation by *S. cerevisiae* during maltose consumption (de Kok et al., 2011), Marques et al. (2018) replaced yeast invertase by an intracellular sucrose phosphorylase from the bacterium *Leuconostoc mesenteroides*. Sucrose phosphorylase converts sucrose and inorganic phosphate into fructose and glucose-1-phosphate (glucose-1P) (Weimberg & Doudoroff, 1954), which can subsequently be isomerised to glucose-6P via the *S. cerevisiae* phosphoglucomutase (Pgm2). By circumventing the ATP-requiring hexokinase reaction for one of the monomers, this phosphorolysis pathway saves one mole of ATP per mole of sucrose consumed. However, since phosphorolysis takes place in the cytosol and uptake of sucrose in yeast involves proton symport via α-glucoside transporters (e.g. Mal11, Mal21, Mal31, Mph2, Mph3) (Badotti et al., 2008; Stambuk et al., 1999), protons have to be exported back to the extracellular space via plasma membrane H^+-ATPase (Pma1), which has a H^+/ATP stoichiometry of 1:1 (Van Leeuwen, Weusthuis, Postma, Van den Broek, & Van Dijken, 1992). For this reason, the overall free-energy conservation in the engineered 'phosphorolytic'

strain did not change relative to a wild-type strain: in both cases, anaerobic fermentation of sucrose yielded 4 mol ATP per mol of sucrose. To gain one additional mol of ATP per mol of sucrose consumed, the native sucrose transporters should be replaced by a transporter that mediates facilitated diffusion (de Kok, Kozak, Pronk, & van Maris, 2012). If the ATP yield of sucrose fermentation by *S. cerevisiae* could be increased to 5 mol of ATP per mole of sucrose, this could theoretically expand the range of products that can be made in anaerobic yeast-based processes. Such anaerobic production processes have considerable cost advantages relative to aerated processes since less power has to be devoted to air compression, reactor agitation and cooling (de Kok, 2012; Weusthuis, Lamot, van der Oost, & Sanders, 2011; Mans, 2017).

Many studies have demonstrated functional expression of heterologous transporters in *S. cerevisiae* (He, Wang, & Yan, 2014; Kim, Lee, Galazka, Cate, & Jin, 2014; Lin et al., 2014; Ton & Rao, 2004). However, efficient transporter sorting, folding and stability in yeast cells can be a major challenge (Froissard et al., 2006; Hernández, 2005). Therefore, functional characteristics of plant transporters expressed in *S. cerevisiae* cannot always be derived from *in planta* results (Bassham & Raikhel, 2000). Few previous studies have investigated the expression of plant sucrose uniporters in yeast (Chen, 2014; Lin et al., 2014; Zhou, Grof, & Patrick, 2014; Zhou, Qu, Dibley, Offler, & Patrick, 2007). Marques et al. (2018) expressed 5 candidate genes for sucrose uniporters in *S. cerevisiae*, of which only one, encoding the *Phaseolus vulgaris* Sucrose Facilitator 1 (*PvSUF1*), supported growth of a strain (after one week time) in which extracellular invertase and sucrose symport have been eliminated. However, the anaerobic specific growth rate on sucrose of this strain was only 0.05 h^{-1}, which was six-fold lower than that of the reference strain CEN.PK113-7D. Indeed, rates of uptake of radiolabelled sucrose by the *PvSUF1*-expressing strain were close to the detection limit. While some YPet-tagged *Pv*Suf1 was found at the yeast plasma membrane, it also accumulated in intracellular compartments, suggesting poor intracellular targeting and/or high turnover of heterologously expressed *Pv*Suf1 (Marques et al., 2018).

In view of the potential relevance of expressing *PvSUF1* and other plant sugar transporter genes in the metabolic engineering of *Saccharomyces cerevisiae*, this study aimed at investigating

genetic factors involved in optimal functional expression of *PvSUF1* in this yeast. To this end, we used laboratory evolution to select for *Pv*Suf1-dependent *S. cerevisiae* strains with improved sucrose-uptake kinetics and analysed causal mutations for improved sucrose consumption by evolved strains. To study the energy coupling of sucrose transport by evolved and unevolved *Pv*Suf1 variants, we analysed sucrose-induced proton-uptake by reference and evolved strains and measured biomass yields of yeast strains expressing different *Pv*Suf1 variants in anaerobic, sucrose-limited chemostat cultures.

MATERIALS AND METHODS

Microbial strains and cultivation medium

The *S. cerevisiae* strains used in this study (**Table 1, Figure 1**) share the CEN.PK genetic background (Entian & Kötter, 2007; Nijkamp et al., 2012). Cultures were grown in an Innova incubator shaker (Eppendorf, Hamburg, Germany) at 200 rpm, 30 °C, in 500 mL shake flasks containing 100 mL of either yeast-peptone-dextrose medium (YPD) or synthetic medium (Verduyn, Postma, Scheffers, & van Dijken, 1992) with 20 g/L glucose as the carbon source (SMD). Frozen stock cultures were prepared by adding glycerol (30 % v/v final concentration) to exponentially growing cells, followed by aseptic freezing and storage of 1 mL aliquots at -80 °C.

Molecular biology techniques

PCR amplifications for strain construction were performed with Phusion Hot Start II High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Diagnostic PCR was carried out using DreamTaq (Thermo Fisher Scientific). The primers used in this study (**Table S1)** where purchased from Sigma-Aldrich. Yeast genomic DNA was isolated using the YeaStar Genomic DNA kit (D2002, Zymo Research, Irvine, CA). DNA fragments obtained by PCR were separated by gel electrophoresis using 1% (w/v) agarose gels

(Thermo Fisher Scientific) in Tris-acetate-EDTA buffer (Thermo Fisher Scientific). DNA fragments were excised from the gels and purified by gel purification kit (D2004, Zymo Research). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich) according to the supplier's manual and from *S. cerevisiae* using Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research). Restriction analysis was performed using FastDigest enzymes (Thermo Fisher Scientific) according to the manufacturer's manual. *E. coli* DH5α cells (18258-012, Thermo Fisher Scientific) were transformed via electroporation using a Gene Pulser Xcell Electroporation System (Bio-Rad), following the manufacturers protocol.

Sanger and whole-genome sequencing

Genome-integrated and episomal expression cassettes present in the evolved strains (IMS644, IMS646, IMS647, IMS648 and IMS649) were Sanger sequenced at BaseClear BV (Leiden, The Netherlands). Primers 6018&7822 (**Table S1**) were used to amplify the *SPase*-expression cassette for sequencing. Similarly, primers 5606&7827 were used to amplify the *PvSUF1* allele of strain IMS648, before Sanger sequencing. Plasmids expressing the *PGM2* and *PvSUF1* genes were extracted from yeast using Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research) and transformed into *E. coli* (DH5α cells, 18258-012, Thermo Fisher Scientific) via electroporation using a Gene Pulser Xcell Electroporation System (Bio-Rad) for propagation. After extraction from *E. coli* using the Sigma GenElute Plasmid kit (Sigma-Aldrich), plasmids were used as a template to sequence the *PGM2* and *PvSUF1* cassettes. Genes were sent for Sanger sequencing using the primers listed in **Table S1** resulting in a two times coverage of each base pair. The promoter and terminator regions sequenced were: 420 bp upstream and 280 bp downstream of the *PvSUF1* ORF, 500 bp upstream and 170 bp downstream of the *PGM2* ORF and 670 bp upstream and 370 bp downstream of the *LmSPase* ORF. Genomic DNA for whole-genome sequencing was extracted using the Qiagen 100/G kit following the manufacturer's protocol (Qiagen, Hilden, Germany), from shake-flask cultures grown on SMD. Whole-genome sequencing was performed by Novogene (HK) Company Ltd (Hong Kong, China). A PCR-free insert library of

350-bp genomic fragments was created and sequenced paired end (150-bp reads). A minimum data quantity of 2600 MB was generated per strain, representing a minimum 216-fold coverage. Data analysis was performed by mapping the sequence reads to the CEN.PK113-7D reference (Salazar et al., 2017) using the Burrows-Wheeler alignment (BWA) tool (Li & Durbin, 2009) and processed with Pilon (Walker et al., 2014). The sequencing data of the parental strain IMZ630 and of the evolved isolates (IMS644, IMS646, IMS647, IMS648 and IMS649) were deposited at NCBI under the BioProject ID: PRJNA471800.

Plasmid construction

Plasmid assembly was performed by *in vivo* homologous recombination in *S. cerevisiae* transformed according to Gietz and Woods (2002) using 0.5 - 1.0 µg of DNA. Construction of the "empty" vector plasmid pUDE690 (**Table 2**) was done by amplifying pUDE486 in two parts, leaving out the *PvSUF1* ORF. Both parts were amplified using primers 11846 & 5975 and 5974 & 11847 (**Table S1**). The ends of the amplified fragments shared 60 bp homology regions and were joined *in vivo* (Kuijpers et al., 2013) by transforming both parts in strain IMZ630 (**Table 1**). Plasmids pUDE544, pUDE546, pUDE547, pUDE559 and pUDE560 were extracted from sucrose-evolved strains IMS644, IMS646, IMS647, IMS648 and IMS649, respectively, and transformed into *E. coli* DH5α cells for plasmid multiplication and storage (**Table 1**, **Table 2)**. *PvSUF1*-expression cassettes from pUDE413 and pUDE486 were replaced by *PvSUF1*-expression cassettes containing mutations in the *PvSUF1* ORF. For this purpose, pUDE413 or pUDE486 backbone was amplified using primers 9041&5975 and assembled *in vivo* with *PvSUF1*-expression cassette amplified from pUDE545 or pUDE546 using primers 2889&10307 in strain IMZ630, resulting in plasmids pUDE565 and pUDE566, respectively, when pUDE413-backbone was used, and plasmids pUDE567 and pUDE568, respectively, when backbone from pUDE486 was used (**Table 2**). pUDE691 was constructed in the same way as pUDE567 and pUDE568 with the exception that, for construction of this plasmid, the *PvSUF1*-expression cassette was amplified from pUDE560 (**Table 2**).

Strain construction

Strain IMZ730 was constructed by transforming pUDE690 into IMZ630 (**Figure 1**). Plasmids present in the evolved strains, IMS644, IMS646, IMS647, IMS648 and IMS649, were removed by overnight cultivation on YPD medium followed by selection on SMD agar plates supplemented with 0.15 g/L uracil and 1 g/L 5'-fluoroorotic acid (Boeke, La Croute, & Fink, 1984), resulting in strains IMS652, IMS653, IMS654, IMS655 and IMS656, respectively (**Figure 1**). Expression cassettes (*TEF1*p-*PvSUF1*-*CYC1*t) were extracted from the evolved strains, cloned into a 2-µm plasmid (with and without *PGM2*) resulting in plasmids pUDE565, pUDE566, pUDE567, pUDE568 and pUDE691, which were subsequently transformed into an unevolved strain background (IMZ630), resulting in strains IMZ712, IMZ713, IMZ714, IMZ715 and IMZ729, respectively. Similarly, the *PvSUF1*-containing plasmids extracted from the evolved strains (pUDE545, pUDE546 and pUDE560) were transformed into an evolved background, IMS656 (obtained after plasmid removal from strain IMS649), resulting in strains IMZ724, IMZ725 and IMZ727, respectively (**Figure 1**).

Cultivation conditions

Shake flask cultivations were carried out in 500 mL flasks containing 100 mL synthetic medium with 20 g/L initial sucrose (SMS) (if not stated, sugar concentration in SMD or SMS was 20 g/L), in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) set at 200 rpm and 30 °C. For growth rate determinations, frozen stock cultures were first inoculated in a shake flask containing SMD. After reaching stationary phase, cultures were transferred to SMS (initial $OD_{660nm} \leq 0.2$) and incubated under an anaerobic atmosphere (5% H_2 6% CO_2 and 89% N_2) in a Bactron X anaerobic chamber (Shell Lab, Cornelius, OR) until exponential growth was observed. Inside the anaerobic chamber, exponentially growing cultures were then transferred to fresh SMS (initial $OD_{660nm} = 0.2$) and samples were taken hourly until stationary phase was reached. Specific growth rates were calculated from at least five OD measurements evenly distributed

over the exponential growth phase. For anaerobic cultivations, synthetic medium was supplemented with 10 mg/L ergosterol and 420 mg/L Tween 80. Since stock solutions of these anaerobic growth factors were prepared with ethanol, the initial ethanol concentration in media for anaerobic growth was 0.67 g/L. Chemostat cultivations were performed in 1.5 L bioreactors (800 rpm, 30°C) (Applikon, Delft, The Netherlands) with 1 L SMS supplemented with 0.15 g/L Antifoam C (Sigma-Aldrich), which was autoclaved separately (120 °C for 20 min) (Verduyn et al., 1992). The culture pH was maintained at 5.0 by automated addition of 2 M KOH. For aerobic cultivation, 500 mL min^{-1} compressed air was sparged in the reactor. To maintain anaerobic conditions, the bioreactors were sparged with 500 mL N_2 min^{-1} (<5 ppm O_2) (also the medium vessels were sparged with N_2) and equipped with Norprene tubing to minimize oxygen diffusion. After the batch phase, medium pumps were switched on, resulting in the continuous addition of SMS containing 25 g/L sucrose to the cultures. The working volume was kept constant at 1.0 L using an effluent pump controlled by an electric level sensor, resulting in a constant dilution rate. The exact working volume and medium flow rate were measured at the end of each experiment. Chemostat cultures were assumed to be in steady state when, after five volume changes, the biomass concentration and the CO_2 production rate varied by less than 4.5 % over at least another 2 volume changes.

Laboratory evolution

Repeated batch cultivation (SBRs) of strains *S. cerevisiae* IMZ636 and IMZ696 was initiated by serial transfers in shake flasks (5 to 11 transfers). Shake flask cultures were grown in an anaerobic chamber with 20 mL SMS in 30 mL shake flasks incubated at 30 °C and 200 rpm. After this initial phase, evolution was continued in N_2-sparged reactors of 500 mL total volume (Infors HT Multifors 2, Infors AG, Switzerland) with 100 mL SMS (50 mL min^{-1} N_2 gas, 400 rpm, 30 °C). The 100 mL working volume was possible due to manufacturer's special modifications on the vessel and jacket size. For strain IMZ636, three evolution lines were carried out in parallel. For IMZ696, two parallel evolution lines were performed. Culture pH was maintained at 5.0 by

automatic addition of 2 mol L^{-1} KOH. Growth rate was estimated from each batch based on the variation of CO_2 concentration in the off-gas. In yeast, CO_2 production is directly proportional to cell concentration in the reactor since the production of this gas is linked to glycolysis activity. Empty-refill cycles were programmed using the Iris 6 bioprocess software (Infors AG, Switzerland). When the off-gas CO_2 concentration achieved 0.4 %, the empty-refilling sequence was started. Such CO_2 concentration was lower than the maximum of 0.9 % that would be produced at the end of the exponential growth phase (this value was measured before starting the empty-refill cycles to better adjust evolution settings), which guarantees that a new cycle was started before the cells entered stationary phase, which otherwise could delay the evolutionary process. In each cycle, 90 % of the medium was substituted by fresh medium. For evolution of strain IMZ696, the cultivation method was changed from SBRs to accelerostat cultivation (Bracher et al., 2017), after the growth rate did not increase further with the SBR strategy. Accelerostat cultivation, which was conducted in the same reactors used for the SBR cultivations, were continuously fed with SMS containing 25 g/L sugar, while culture liquid was removed to keep the working volume constant. The dilution rate, which was initially set at 0.09 h^{-1}, was automatically increased in response to the CO_2 concentration in the off-gas. This means that the speed of the feed pump (thus the dilution rate) was increased or decreased if the off-gas CO_2 profile showed consistent increase or decrease over a period of 24 h. Four single-colony isolates from each evolution line were obtained by restreaking thrice on plates containing selective SMS medium, which were incubated anaerobically at 30 °C. To prepare frozen stock cultures of sucrose-evolved strains under selective conditions, cells from a single colony were inoculated in 20 mL liquid SMS medium in 30 mL shake flasks, which were incubated under anaerobic conditions (Bactron X anaerobic chamber, Shell Lab, Cornelius, OR, 200 rpm, 30 °C). Based on the anaerobic growth profile of the four single colony isolates on SMS (e.g. shortest lag phase and/or highest growth rate), one colony was selected and named as representative of the correspondent evolution line. Stocks in SMD were also prepared, to be used in characterization experiments, such as measurement of the growth rate on sucrose-based medium.

Analytical methods

Optical density of cultures at 660 nm was monitored using a Libra S11 spectrophotometer (Biochrom, Cambride, United Kingdom). Culture dry weight measurements and rapid quenching of culture samples were carried out as described by Marques et al. (2018). HPLC analysis of the supernatant and residual sugar determination were performed as described previously (de Kok et al., 2011; Marques et al., 2017). Off-gas CO_2 concentrations were measured with an NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO).

Proton translocation assay

Cells used in proton translocation assays were harvested from aerobic sucrose-limited chemostat cultures. The same culture conditions as described for anaerobic chemostats above were used, with the following changes: reactors were sparged with 500 mL min^{-1} air, the sucrose concentration in the medium vessel was decreased to 7.5 g/L, the dilution rate was set at 0.03 h^{-1} and Tween 80 and ergosterol were omitted from the medium. After 5 volume changes, when CO_2 concentrations in the off-gas were stable, cells were harvested by centrifugation at 5 000 x *g* for 5 min, at room temperature, washed with distilled water and resuspended in potassium phthalate buffer (1.25 mM, pH 5) to a final concentration of 12 g dry weight L^{-1}. 5 mL of the cell suspension were incubated at 30 °C in a magnetic stirred vessel with a S220 SevenCompact™ pH/Ion electrode attached (Mettler Toledo, Greifensee, Switzerland). After stabilization of the pH signal, 100 µL of a 1 mol L^{-1} sugar (sucrose, maltose, glucose or fructose) solution was added (final concentration 20 mM) and changes in the pH were recorded using the LabX™ pH Software (Mettler Toledo, Greifensee, Switzerland). Calibration was performed by addition of 5 µL aliquots of 10 mM NaOH to the cell suspensions.

RESULTS

Increased growth rates of *PvSUF1*-dependent yeast strains after laboratory evolution in a sucrose-based medium

In the engineered *S. cerevisiae* strain IMZ636 (*malΔ mphΔ suc2Δ imaΔ sga1Δ LmSPase PvSUF1*), sucrose metabolism is strictly dependent on uptake via the plant transporter *Pv*Suf1 and subsequent intracellular cleavage by a bacterial sucrose phosphorylase. In anaerobic shake flask cultures, strain IMZ636 grew on SMS (synthetic medium with 20 g/L sucrose as sole carbon source) at a specific growth rate of 0.05 h^{-1} (**Table 3**). This growth rate is much lower than that of the reference strain CEN.PK113-7D (0.29 h^{-1}, **Table 3**). To select for faster growth on sucrose, strain IMZ636 was grown in anaerobic sequencing-batch bioreactors (SBR). In three independent evolution lines, the specific growth rate increased from 0.05 h^{-1} to 0.10-0.17 h^{-1} (estimated from off-gas CO_2 profiles) after 56-80 cycles (approximately 150 generations, **Figure 2**). When no further increase in the growth rate was observed, four single colonies were isolated from each experiment and characterized in anaerobic shake-flask cultures on SMS. Differences in growth rate among the four colonies isolated from each evolution line indicate heterogeneity in the evolved population (results not shown). The fastest isolate of each evolution line displayed specific growth rates of 0.07 h^{-1} (IMS644 from reactor "A"), 0.09 h^{-1} (IMS646 from reactor "B") and 0.08 h^{-1} (IMS647 from reactor "C") (**Table 3**). These growth rates were consistently higher than those of the unevolved IMZ636 strain, but differed slightly from those estimated from CO_2 profiles in the SBR experiments. These differences may have been caused by the different methods used to access growth rate (optical density measurements versus on-line CO_2 data; shake flasks versus bioreactors). Additionally, the shake-flask experiments were performed with single cell lines while the specific growth rates estimated from the SBR experiments represented growth of an evolving and probably heterogeneous population.

The anaerobic specific growth rates on sucrose of the evolved, IMZ636-derived strains were still 3-4 fold lower than that of the congenic reference strain CEN.PK113-7D (**Table 3**). Marques et al. (2018) showed that overexpression of phosphoglucomutase (*PGM2*) in a unevolved *PvSUF1-SPase*-expressing strain increased its growth rate from 0.05 to 0.07 h^{-1} (IMZ696). To investigate

whether in the laboratory evolution experiments, specific growth rates were limited by phosphoglucomutase levels, a new sequential batch cultivation evolution was initiated with a *PGM2*-expressing strain (IMZ696-*PvSUF1, SPase, PGM2*). In two independent evolution lines, the specific growth rate on sucrose increased from 0.07 to 0.15-0.20 h^{-1} (calculated from the off-gas CO_2 concentration from each reactor) after 52 cycles (ca. 120 generations in reactor A and 190 generations in reactor B, **Figure 3A** and **3B**). Since no further increase in growth rate was observed in this reactor's configuration, laboratory evolution was continued using accelerostat cultivation, with an initial dilution rate of 0.09 h^{-1}. In accelerostats, the selective pressure is on growth rate but also on substrate affinity (Bracher et al., 2017). The feed rate was automatically increased or decreased based on on-line analysis of the CO_2 concentration in the off-gas. After 20-30 days (approximately 130 generations), the dilution rate in the two reactors had increased to 0.25 h^{-1} and 0.17 h^{-1} (**Figures 3C** and **3D**), while the residual sucrose concentration had decreased from 8 g/L to approximately 2.5 g/L after accelerostat selection (results not shown). These dilution rates were close to the anaerobic specific growth rate on sucrose reported for a *Lm*SPase-dependent strain expressing the native *MAL11* sucrose symporter (0.23 h^{-1}, Marques et al. 2018). Single colony isolates from each reactor (IMS648 from reactor shown in **Figure 3A** and IMS649 from reactor shown in **Figure 3B**) exhibited specific growth rates of 0.19 h^{-1} and 0.23 h^{-1}, respectively (**Table 3**). These specific growth rates were close to that of IMZ709 (*MAL11, SPase, PGM2*), and almost three times higher than that of the unevolved parental strain IMZ696 (**Table 3**).

Sucrose/H^+ symport activity of evolved *Pv*Suf1 transporters as revealed by proton-uptake studies and biomass yields on sucrose

Although *PvSUF1* has been reported to encode a sucrose uniporter (Zhou et al., 2007), a recent study (Marques et al. 2018) on the expression of *PvSUF1* in *S. cerevisiae* casts doubt on the actual mechanism of sucrose transport: sucrose-dependent proton uptake rates of 8.2 ± 2.2 μmol H^+ (g biomass)$^{-1}$ min^{-1} were measured with cell suspensions of *S. cerevisiae* strain IMZ696 (*PvSUF1,*

SPase, PGM2) grown in aerobic, sucrose-limited chemostat cultures (**Figure 4**, data extracted from Marques et al. 2018). This measured uptake rate would in principle be more than sufficient to account for all sucrose uptake in the aerobic chemostat cultures. If we considered the residual sucrose concentration in the bioreactor negligible, a specific sucrose uptake rate of 3.7 ± 0.1 µmol sucrose (g biomass)$^{-1}$ min^{-1} would be calculated. In replicate chemostat experiments with strain IMZ696, different mutations were found in *PvSUF1 (Pv*Suf1^{T302I} in one culture and *Pv*Suf1$^{E308K\ V323F}$ in the other). Since the frozen stock cultures from which the chemostats were inoculated did not contain mutations in *PvSUF1*, these mutations probably conferred a selective advantage during sucrose-limited chemostat cultivation (Marques et al. 2018).

All evolved strains (IMS644, IMS646, IMS647, IMS648 and IMS649) obtained in the present study displayed sucrose-induced H^+ uptake (**Figure 4**). Strain IMS644 showed an initial H^+ uptake rate that was close to that of the unevolved parental strain IMZ696 (13 ± 3.7 µmol H^+ min^{-1} (g cell)$^{-1}$ for IMS644 vs. 8 ± 2.2 µmol H^+ min^{-1} (g cell)$^{-1}$ for IMZ696) (**Figure 4**), but all other strains showed an at least three-fold higher H^+ uptake rate compared to IMZ696 (25-40 µmol H^+ min^{-1} (g cell)$^{-1}$ vs. 8 ± 2.2 µmol H^+ min^{-1} (g cell)$^{-1}$ for IMZ696) (**Figure 4**). These high rates of sucrose-dependent proton uptake approached those of a reference strain expressing the *S. cerevisiae* Mal11 proton symporter (52 ± 8.7 µmol H^+ min^{-1} (g cell)$^{-1}$ for IMZ709, data from Marques et al. (2018) **(Figure 4**). The evolved *PvSUF1*-expressing strains exhibited similar proton uptake rates with maltose as with sucrose, while no proton uptake was observed upon addition of fructose or glucose. Consistent with literature reports (Wieczorke et al., 1999) the control strain IMZ709 (*MAL11, SPase*) exhibited glucose-dependent proton uptake (data from Marques et al. 2018) (**Figure 4**).

An alternative way to investigate energy coupling of disaccharide uptake in *S. cerevisiae* is to measure biomass yields in anaerobic, disaccharide-limited chemostat cultures (de Kok, 2012). If sucrose uptake occurs via symport with a single proton, one ATP molecule has to be consumed by the plasma membrane H^+/ATPase Pma1 to expel the symported proton. On the contrary, if sucrose uptake is passive, no ATP will be consumed. This difference of 1 ATP has a high impact

on the biomass yield on sugar under anaerobic conditions, which can be precisely determined in anaerobic chemostats (de Kok et al., 2011; Marques et al., 2018; Verduyn, Postma, Scheffers, & van Dijken, 1990).

The strains used in this study cleaved sucrose intracellularly via phosphorolysis. In such strains, expression of a sucrose/H^+ symporter should result in a net generation of 4 mol ATP per mol sucrose under anaerobic conditions. If sucrose uptake occurred by uniport, this ATP yield would change to 5 ATP/sucrose, a 25% increase (Marques et al., 2018). In sucrose-limited, anaerobic chemostat cultures, a 25% increase of the ATP yield from sucrose dissimilation should result in a 25% increase in the biomass yield on sucrose (Verduyn et al., 1990). Two of the evolved strains (IMS646 from the evolution started with IMZ636 and IMS649 from the evolution started with IMZ696) were characterized in chemostat cultures and their biomass yields were compared to those displayed by reference strains (IMZ665 and IMZ709), which both expressed *MAL11* instead of *PvSUF1*. No differences in biomass yield were observed between a *MAL11* expressing strains and strains evolved with *PvSUF1*: the observed biomass yield of strain IMZ665 (*MAL11, SPase*) was 0.086 ± 0.002 g (g glucose equivalent)$^{-1}$ while that of IMS646 (*PvSUF1, SPase*) was 0.082 ± 0.004 g (g glucose equivalent)$^{-1}$ (**Table 4** and **Table S2**). Similarly, for the strains expressing *PGM2* (IMZ709 and IMS649) the biomass yield was not higher in cultures of the evolved *PvSUF1*-expressing strain (0.087 ± 0.000 g (g glucose equivalent)$^{-1}$ for IMS649) than in cultures of the *MAL11*-expressing strain (0.091 ± 0.006 g (g glucose equivalent)$^{-1}$ for IMZ709) (**Table 4** and **Table S2**).

Evolved strains contain mutations in *PvSUF1*

Non-conservative single-nucleotide mutations were detected in the *PvSUF1* open reading frames of all *Pv*Suf1-dependent strains evolved for faster growth on sucrose (IMS644, IMS646, IMS647, IMS648 and IMS649; **Figure 5**). No mutations were found in the promoter (*TEF1*) or in the terminator (*CYC1*) regions of the *PvSUF1* expression cassettes. Strains IMS644 and IMS647, which were independently evolved from strain IMZ636 (*PvSUF1,SPase*), contained the same

mutation (*PvSUF1*$^{\text{YAAGSFSG-duplication}}$): a tandem duplication of 8 amino acids that, based on amino-acid hydrophobicity plots (*Protter* algorithm, (Omasits, Ahrens, Mu, & Wollscheid, 2014)), was predicted to be localized partially in the extracellular surface of trans-membrane domain 5 (TM5) and in the loop connecting TM5 to TM6 (loop 5/6) (**Figure 5** and **Figure 6**). Strain IMS646, which was also evolved from IMZ636 (*PvSUF1, SPase*), contained a mutation that resulted in a substitution of glycine 326 (TM8) for a cysteine (G326C) (**Figure 5**). Evolved strain IMS649, derived from the IMZ696 strain (*PvSUF1, SPase, PGM2*), carried a combination of the abovementioned *PvSUF1*$^{\text{G326C}}$ mutation and two additional mutations. One of these caused an I209F substitution in the loop connecting TM5 to 6 (loop 5/6) at the extracellular surface while the other led to a C265F substitution, positioned in loop 6/7 on the cytosolic side (**Figure 5** and **Figure 6**). The *PvSUF1* allele of the remaining strain, which was evolved from IMZ696 (*PvSUF1, SPase, PGM2*), IMS648, contained three mutations, leading to Y128C, C228G, and G457D substitutions (**Figure 5**). In this strain, the *PvSUF1*- and *PGM2*-expression cassettes were no longer located on the original 2µ-expression vector. Instead, both cassettes were found to be integrated into chromosomal DNA. Accordingly, strain IMS655, which was obtained by curing the 2µ-plasmid from strain IMS648, retained its ability to grow on SMS (**Figure S1**). The other four evolved strains did not grow on sucrose after plasmid removal (IMS652, IMS653, IMS654 and IMS656). No mutations were found in the *PGM2* or *SPase* expression cassettes of any of the evolved strains.

Independently evolved strains in a sucrose-based medium show common whole-chromosome and segmental aneuploidies

Besides the mutations found in *PvSUF1*, the evolved strains also showed duplication of chromosomes and/or chromosomal segments. Whole-genome sequencing revealed duplication of the right arm of chromosome 14 (after position ~500,000) in three independently evolved strains: IMS644, IMS646 and IMS649 (**Table 5, Figure S2**). Similarly, a segmental duplication of chromosome 16 (right arm, after position ~800,000) was found in strains IMS646 and IMS649.

A central region of chromosome 13 (position ~300,000 to ~350,000) was triplicated in strains IMS644 and IMS647. Strain IMS644 showed a complete duplication of chromosome 1, as well as of the right arm of chromosome 2 (after position ~600,000; (**Table 5, Figure S2**). To investigate whether these mutations contribute to the phenotype, reverse engineering of the mutated *PvSUF1* alleles was carried out both in an unevolved and in an evolved strain background, as detailed in the next section.

Reverse engineering of evolved *PvSUF1* alleles enables fast growth on sucrose

To investigate whether the faster growth on sucrose observed after evolution of *PvSUF1*-expressing strains could be exclusively attributed to the mutations found in *PvSUF1*, *PvSUF1* expression cassettes (*TEF1*p-*PvSUF1*-*CYC1*t) were first isolated from each of the evolved strains (IMS646, IMS647 and IMS649) and cloned into a 2-µm plasmid. Two versions were constructed, one carrying an additional *PGM2*-expressing cassette and another one without it. The resulting plasmids were transformed into an unevolved background strain, IMZ630 (*malΔ mphΔ suc2Δ imaΔ SPase*), resulting in strains IMZ712-715 and IMZ729 (**Table 6**). Strain IMZ712 (expressing *PvSUF1*^{G326C}) grew anaerobically on sucrose at 0.05 h^{-1} (**Table 6**), which corresponds to the specific growth rate displayed by the unevolved parental strain (IMZ636, **Table 3**). Co-expression of *PGM2* did not lead to a higher growth rate (IMZ714, µ = 0.05 h^{-1}, **Table 6**). Overexpression of a *PvSUF1*$^{YAAGSFSG\text{-duplication}}$ variant that encoded the 8 amino-acid duplication described above (from strain IMS647) in the unevolved background also, by itself, did not result in an increased specific growth rate (IMZ713 µ = 0.05 h^{-1}) (**Table 6**). However, when this mutation was combined with the overexpression of *PGM2* (IMZ715), a specific growth rate of 0.10 h^{-1} was observed (**Table 6**). The *PvSUF1* allele derived from strain IMS649, the evolved strain that grew at 0.20 h^{-1} (**Table 3**), supported high specific growth rates upon introduction in a unevolved background when combined with *PGM2* overexpression (strain IMZ729, µ = 0.17 ± 0.02 h^{-1}) (**Table 6**).

To investigate the possible impact of mutations outside *PvSUF1*, the *PvSUF1* expression plasmid was cured from the fastest growing evolved strain IMS649. The resulting strain (IMS656) was then directly transformed with the vectors extracted from the evolved strains carrying *PvSUF1* variants (*PvSUF1*^{G326C}, *PvSUF1*$^{YAAGSFSG\text{-}duplication}$ and *PvSUF1*$^{I209F\ C265F\ G326C}$) yielding strains IMZ724, IMZ725 and IMZ727, respectively. The growth rates of these strains (0.07 h^{-1}, 0.06 h^{-1} and 0.17 h^{-1}, respectively) were similar to that of the strains expressing evolved *PvSUF1* in an unevolved background **(Table 6)**. This result shows that the improved growth of strain IMS649 was predominantly due to mutations in *PvSUF1* and that other chromosomal alterations had at most a small impact on the strain's specific growth rate on sucrose (**Table 6**).

DISCUSSION

Optimal expression of heterologous transporters in yeast can be hampered by protein misfolding, incorrect sorting, cell toxicity due to protein accumulation in intracellular compartments (unfolded protein response - UPR), rapid endocytosis and turnover, among other phenomena (Bassham & Raikhel, 2000; Froissard et al., 2006; Hernández, 2005; Nielsen, 2013). In this study, genetic factors involved in the efficient expression of a putative plant sucrose uniporter (*Pv*Suf1) in *S. cerevisiae* were identified by a combination of laboratory evolution, whole genome sequencing, reverse engineering of mutations observed in evolved strains and physiological analysis of evolved and reverse engineered strains.

Laboratory evolution of yeast dependent on sucrose uptake via *Pv*Suf1 resulted in faster growing strains – when compared to the parental ones – which contained mutations in the *PvSUF1* allele (**Figure 5**). Besides faster growth, the mutations may also be responsible for lowering the residual sucrose concentration in the accelerostat reactor (from 8 to 2.5 g/L, see Results section). Chromosomal duplications were also found in the genome of the evolved strains (**Table 5**, **Figure S2**) affecting genes that encode proteins involved in transporter sorting, ubiquitination and degradation: *COS10, SEC12 and SIS1*, which are present on chromosome 14 (Luke, Sutton, & Arndt, 1991; Macdonald et al., 2015; Nakano, Brada, &

Schekman, 1988); *CUR1* and *SEC23* from chromosome 16 (Alberti, 2012) and *UBC7* found on chromosome 13 (Hiller, Finger, Schweiger, & Wolf, 1996). However, these genomic alterations are unlikely to be the main cause of the improved specific growth rate of the evolved strains since reverse engineering of a mutated version of *PvSUF1* in unevolved *S. cerevisiae* sufficed to generate a strain that grew as fast as the fastest-growing evolved strain (**Table 6**). Among the mutations found in *PvSUF1*, those involving cysteine residues predominated (*PvSUF1*^{G326C}, *PvSUF1*$^{G457D\ Y128C\ C228G}$, *PvSUF1*$^{I209F\ C265F\ G326C}$; **Figure 5**). Correct formation of disulfide bonds has previously been reported to be important for inter- and intramolecular interactions of SUT (sucrose transporter) proteins and sucrose uptake activity (Krügel et al., 2008, 2012). *Pv*Suf1 contains only 3 out of the 4 cysteine residues conserved in all other plant sucrose transporters (**Figure 5**) (Lemoine, 2000). Since the *PvSUF1*^{G326C} mutation was found in two independently evolved strains (IMS649 and IMS646), presence of a cysteine in position 326 of *Pv*Suf1 may be important for protein folding and oligomerization in yeast, but further research is clearly needed to test this hypothesis.

Sugar-proton symport assays showed that all *PvSUF1*-expressing *S. cerevisiae* strains tested (including those that were not subjected to laboratory evolution) displayed at least some sucrose/H^+ symport activity, which was high enough to explain their observed rates of sucrose uptake (**Figure 4**). While, based on heterologous expression, we cannot draw definitive conclusions on energy coupling of wild type *Pv*Suf1-mediated sucrose transport, our results warrant reinvestigation of *Pv*Suf1's mechanism in a plant context.

The present study shows that laboratory evolution is a robust methodology to improve the expression of sucrose transporters in yeast and that mutations on the transporter itself are sufficient for its efficient expression meaning that alterations in the host genome are not necessary. Four out of six observed amino acid substitutions encoded by evolved *PvSUF1* alleles removed or introduced a cysteine residue, this demonstrates that engineering cysteine residues might be a rational way to improve the expression of plant sugar transporters in yeast. Study of the mutations identified in this work in different transporters, preferably combined with

structural information, will identify underlying mechanisms and increase the predictability of functional expression of heterologous transporters in yeast.

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Conflict of interest. None declared

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672

673

Table 1: *S. cerevisiae* strains used in this study.

Strain	Relevant genotype	Parental strain	Source
CEN.PK113-7D	MATa *URA3 LEU2 MAL2-8^{C} SUC2*		Entian and Kötter, 2007
IMZ630	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase*		Marques et al. 2018
IMZ730	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE690 (*URA3 PGM2*)	IMZ630	This study
IMZ636	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE413 (*URA3 PvSUF1*)	IMZ630	Marques et al. 2018
IMS644	Single colony isolate from evolution line "A"	IMZ636	This study
IMS646	Single colony isolate from evolution line "B"	IMZ636	This study
IMS647	Single colony isolate from evolution line "C"	IMZ636	This study
IMZ696	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE486 (*URA3 PvSUF1 PGM2*)	IMZ630	Marques et al. 2018
IMS648	Single colony isolate from evolution line "A"	IMZ696	This study
IMS649	Single colony isolate from evolution line "B"	IMZ696	This study
IMS652	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* Plasmid cured	IMS644	This study
IMS653	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* Plasmid cured	IMS646	This study
IMS654	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* Plasmid cured	IMS647	This study
IMS655	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase PvSUF1::PGM2** Plasmid cured	IMS648	This study
IMS656	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* Plasmid cured	IMS649	This study
IMZ712	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE565 (*URA3 PvSUF1*^{G326C})	IMZ630	This study
IMZ713	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE566 (*URA3 PvSUF1* $^{YAAGSFSG\text{-}duplication}$)	IMZ630	This study
IMZ714	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE567 (*URA3 PvSUF1*^{G326C}*::PGM2*)	IMZ630	This study
IMZ715	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE568 (*URA3 PvSUF1* $^{YAAGSFSG\text{-}duplication}$*::PGM2*)	IMZ630	This study
IMZ729	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE691 (*URA3 PvSUF1*$^{I209F\text{-}C265F\text{-}G326C}$ *PGM2*)	IMZ630	This study
IMZ724	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE545 (*URA3 PvSUF1*^{G326C})	IMS656	This study
IMZ725	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE546 (*URA3 PvSUF1* $^{YAAGSFSG\text{-}duplication}$)	IMS656	This study
IMZ727	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE560 (*URA3 PvSUF1*$^{I209F\text{-}C265F\text{-}G326C}$ *PGM2*)	IMS656	This study
IMZ665	MATa ura3-52 LEU2 MAL2-8^{C} *malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE432 (*URA3 MAL11*)		Marques et a 2018
IMZ709	MATa ura3-52 LEU2 MAL2-8^{C} *malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE496 (*URA3 MAL11 PGM2*)		Marques et a 2018

*"*PvSUF1::PGM2"* expression cassette migrated from the plasmid to a chromosome. The exact site of integration was not investigated in this work.

679 **Table 2: Plasmids used in this study**

Name	Relevant characteristics	Origin
pUDE413	*2μ URA3 pTEF1-PvSUF1-tCYC1*	Marques et al. 2018
pUDE486	*2μ URA3 pTEF1-PvSUF1-tCYC1 pTPI1-PGM2-tTEF1*	Marques et al. 2018
pUDE690	*2μ URA3 pTPI1-PGM2-tTEF1*	This study
pUDE544	Evolved plasmid from IMS644	This study
pUDE545	Evolved plasmid from IMS646	This study
pUDE546	Evolved plasmid from IMS647	This study
pUDE559	Evolved plasmid from IMS648	This study
pUDE560	Evolved plasmid from IMS649	This study
pUDE565	*2μ URA3 pTPI1-PvSUF1*^{G326C}*-tCYC1*	This study
pUDE566	*2μ URA3 pTPI1-PvSUF1*$^{YAAGSFSG\text{-}duplication}$*-tCYC1*	This study
pUDE567	*2μ URA3 pTPI1-PvSUF1*^{G326C}*-tCYC1 pTPI1-PGM2-tTEF1*	This study
pUDE568	*2μ URA3 pTPI1-PvSUF1*$^{YAAGSFSG\text{-}duplication}$*-tCYC1 pTPI1-PGM2-tTEF1t*	This study
pUDE691	*2μ URA3 pTPI1-PvSUF1*$^{I217F,\ C265F,\ G326C}$*-tCYC1 pTPI1-PGM2-tTEF1*	This study

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681

682 **Table 3**: Specific growth rates of unevolved and evolved *S. cerevisiae* strains grown in shake
683 flask cultures containing 20 mL SMS (initial pH 6, 30 °C, 200 rpm) in an anaerobic chamber.
684 Averages and mean deviations were obtained from duplicate experiments. *SPase* was integrated
685 in the genome (*SGA1* locus), while *PvSUF1* was expressed in a 2µ-plasmid with or without *PGM2*.

Strain	**Relevant characteristics**	**Specific growth rate (h^{-1})**
CEN.PK113-7D	*SUC2*	0.29 ± 0.00
IMZ636	*SPase, PvSUF1*	0.05 ± 0.01
IMS644	*SPase, PvSUF1*, evolved "A"	0.07 ± 0.00
IMS646	*SPase, PvSUF1*, evolved "B"	0.09 ± 0.01
IMS647	*SPase, PvSUF1*, evolved "C"	0.08 ± 0.01
IMZ696	*SPase, PvSUF1-PGM2*, parental strain	0.07 ± 0.01
IMS648	*SPase, PvSUF1*-PGM2*, evolved "A"	0.18 ± 0.01
IMS649	*SPase, PvSUF1-PGM2*, evolved "B"	0.19 ± 0.01

686 * *PvSUF1* was found in the genome of this strain, not in the plasmid as in the parental IMZ696.
687

688 **Table 4:** Biomass yields on sucrose and rates of sucrose uptake by *S. cerevisiae* strains grown in
689 anaerobic sucrose-limited chemostat cultures (pH 5, 30 °C, 800 rpm, 500 mL N_2/min). A dilution
690 rate of 0.07 h^{-1} was used for strains IMZ665 (*MAL11, SPase*) and IMS646 (*PvSUF1, SPase*), while
691 0.15 h^{-1} was used for strains IMZ709 (*MAL11, SPase, PGM2*) and IMS649 (*PvSUF1, SPase, PGM2*).
692 Cultures were grown on SMS with 25 g/L sucrose in the feeding medium. Averages and mean
693 deviations were obtained from duplicate experiments. Additional physiological data are
694 provided in **Table S2**.

Strain	**IMZ665 (control)**	**IMS646 (evolved)**	**IMZ709 (control)**	**IMS649 (evolved)**
Relevant genotype	*MAL11 LmSPase*	*PvSUF1 LmSPase*	*MAL11 LmSPase PGM2*	*PvSUF1 LmSPase PGM2*
Actual dilution rate (h^{-1})	0.070 ± 0.000	0.071 ± 0.001	0.147 ± 0.001	0.152 ± 0.001
Biomass yield (g g glucose equivalent^{-1})	0.086 ± 0.002	0.082 ± 0.004	0.091 ± 0.006	0.087 ± 0.000
$q_{sucrose}$ (mmol/g biomass/h)	-2.26 ± 0.06	-2.40 ± 0.06	-4.5 ± 0.3	-4.83 ± 0.04
Residual sucrose (g/L)	0.08 ± 0.02	1.90 ± 1.18	2.03 ± 0.15	4.33 ± 0.80
Carbon recovery (%)	101 ± 1	95 ± 4	105 ± 7	102 ± 1

695

696

Table 5: Summary of whole-chromosome and segmental aneuploidies found in *PvSUF1*-expressing strains evolved on sucrose-based medium relative to the unevolved reference strain IMZ630. "Chr": chromosome. "+": presence of copy number variation. Chromosome positions are shown between brackets.

Strain	Chr1 - Entirely duplicated	Chr2 - Duplication of right arm (600,000 - telomere)	Chr13 - Triplication of short region (300,000 - 350,000)	Chr14 - Duplication of right arm (500,000 - telomere)	Chr16 - Duplication of right end (800,000 - telomere)
IMS644	+	+	+	+	
IMS646				+	+
IMS647			+		
IMS648					
IMS649				+	+

703 **Table 6:** Specific growth rates on sucrose in anaerobic shake flask cultures on SMS of *S.*
704 *cerevisiae* strains (*malΔ mphΔ suc2Δ imaΔ SPase*) expressing different evolved *PvSUF1* alleles.
705 Averages and mean deviations were derived from two biological replicates for each strain.

Strain	**Parental strain**	***PvSUF1* from evolved strain**	**Relevant characteristic**	**Specific growth rate** (h^{-1})
IMZ712	IMZ630	IMS646	*SPase, PvSUF1*^{G326C}	0.05 ± 0.01
IMZ713	IMZ630	IMS647	*SPase, PvSUF1*$^{YAAGSFSG\text{-}duplication}$	0.05 ± 0.01
IMZ714	IMZ630	IMS646	*SPase, PvSUF1*^{G326C} *PGM2*	0.05 ± 0.01
IMZ715	IMZ630	IMS647	*SPase, PvSUF1* $^{YAAGSFSG\text{-}duplication}$ *PGM2*	0.10 ± 0.01
IMZ729	IMZ630	IMS649	*SPase, PvSUF1*$^{I209F\ C265F\ G326C}$ *PGM2*	0.17 ± 0.02
IMZ724	IMS656	IMS646	*SPase, PvSUF1*^{G326C}	0.07 ± 0.01
IMZ725	IMS656	IMS647	*SPase, PvSUF1*$^{YAAGSFSG\text{-}duplication}$	0.06 ± 0.01
IMZ727	IMS656	IMS649	*SPase, PvSUF1*$^{I209F\ C265F\ G326C}$ *PGM2*	0.18 ± 0.01

706

707

708 **LEGENDS TO FIGURES**

709

710 **Figure 1:** Strains construction scheme. Each blue box represents a different strain. Plasmids
711 used are indicated on the top of the corresponding strain. The three main methods of generating
712 strains are shown in the panels: "Evolution", "Plasmid removal" and "Reverse engineering".
713

714 **Figure 2:** Laboratory evolution in sequential batch cultures of *S. cerevisiae* IMZ636 (*PvSUF1,*
715 *SPase*). After 5-11 serial transfers in shake flask cultures, incubated in an anaerobic chamber,
716 evolution was continued in sequential batch bioreactors sparged with N_2 gas. Evolution was
717 conducted independently in triplicate (panels **A**, **B** and **C**). Specific growth rates were estimated
718 from the corresponding CO_2 profiles (not shown here) in the off-gas. 100 mL of SMS were used
719 (pH 5.0, 30 ºC, 250 rpm). Evolution was stopped after approximately 160, 170 and 150
720 generations (panels **A**, **B** and **C**).

721

722 **Figure 3:** Laboratory evolution of *S. cerevisiae* IMZ696 (*PvSUF1, SPase, PGM2*) for faster
723 anaerobic growth on sucrose. After eleven serial transfers in shake flasks in an anaerobic
724 chamber, evolution was continued in bioreactors sparged with N_2 gas, first operated as
725 sequential batch reactors (SBRs) (panels **A** and **B**) and subsequently as accelerostats (panels **C**
726 and **D**) as described in the Methods section. **C, D** (accelerostat): feed and effluent pumps were
727 turned on at 1 % of the maximum speed (equivalent to 0.09 h^{-1} dilution rate). Feed pump speed
728 (black line) was increased automatically based on the CO_2 concentration in the off-gas (blue
729 line). A maximum pump speed corresponding to 0.25 h^{-1} dilution rate was reached in one
730 reactor (**C**) and of 0.17 h^{-1} in the other one (**D**). Laboratory evolution in bioreactors was
731 performed with 100 mL of synthetic medium containing 20 g/L sucrose (batches) and 25 g/L
732 (accelerostat), pH 5, 30 ºC, 250 rpm). Panels **A** and **C** correspond to one evolution line
733 independent from another replicate (panels **B** and **D**). Both evolution lines were started with the
734 same parental strain.
735

736 **Figure 4:** Proton uptake rate of unevolved *S. cerevisiae* IMZ696 (*PvSUF1,SPase,PGM2*), evolved
737 strains (IMS644, IMS646, IMS647, IMS648 and IMS649) and the control strain IMZ709 (*MAL11,*
738 *SPase, PGM2*). Cells were harvested from aerobic sucrose-limited chemostat cultures (D = 0.03 h^{-1},
739 30 ºC, pH 5.0), washed and immediately tested for proton uptake upon addition of sucrose
740 (black bar), maltose (grey bar), fructose (*: H^+ uptake not detected) or glucose (white bar, H^+

uptake induced only in strain IMZ709). 20 mM sugar (final concentration) was added to a K-phthalate suspension (pH 5.0) containing 2.5 g/L cells (30 ºC). Average and mean deviation were obtained from two biological replicates with three experimental replicates each. Calibration was performed as described in the Methods section. Results from strains IMZ696 and IMZ709 were previously published by Marques et al. (2018).

Figure 5: Mutations found in *PvSUF1* after laboratory evolution on sucrose-based medium. Mutated amino acids are highlighted in red and the corresponding strain numbers are indicated. "AA": amino acids. Arrows indicate the location of four conserved cysteine residues in plant sucrose transporters. The cysteine at loop 2/3 is substituted by a serine in the wild-type *Pv*Suf1 protein. Membrane insertion of *Pv*Suf1 was predicted with the *Protter* algorithm (Omasits et al., 2014).

Figure 6: Model of the tertiary structure of wild-type *Pv*Suf1. Extracellular loops are shown at the top and intracellular loops at the bottom of the figure "**A**". View from the extracellular space is shown in "**B**". The 8 amino-acid region in loop 5/6 that was duplicated in strains IMS644 and IMS647 is shown in red. In the same loop, isoleucine 209, which was mutated to phenylalanine (I209F) in strain IMS649 is highlighted in yellow. Images were prepared with PyMOL™ (version 1.7.4.5 Educational Product, Schrodinger, LLC).