

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

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

4

5 Wesley Leoricy Marques<sup>a,b</sup>, Lara Ninon van der Woude<sup>a</sup>, Marijke A. H. Luttik<sup>a</sup>, Marcel van den  
6 Broek<sup>a</sup>, Janine Margriet Nijenhuis<sup>a</sup>, Jack T. Pronk<sup>a</sup>, Antonius J. A. van Maris<sup>a,1</sup>, Robert Mans<sup>a</sup>,  
7 Andreas K. Gombert<sup>b,\*</sup>

8

9 <sup>a</sup> Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ  
10 Delft, The Netherlands

11 <sup>b</sup> School of Food Engineering, University of Campinas, Rua Monteiro Lobato 80, Campinas-SP  
12 13083-862, Brazil

13 <sup>1</sup>Current address: Department of Industrial Biotechnology, School of Engineering Sciences in  
14 Chemistry, Biotechnology and Health, KTH Royal Institute of Technology, AlbaNova University  
15 Center, SE 106 91 Stockholm, Sweden

16 \*corresponding author. Email: gombert@unicamp.br

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20 **ABSTRACT**

21 Knowledge on the genetic factors important for efficient expression of plant transporters in  
22 yeast is still very limited. Here, we investigated if laboratory evolution could improve the  
23 expression a sucrose transporter in yeast and which mutations were crucial for the evolved  
24 phenotype. *PvSuf1* (*Phaseolus vulgaris* sucrose facilitator 1) was key in a previously published  
25 strategy aimed at increasing ATP yield in *S. cerevisiae* since it has been described as an  
26 uniporter. However, attempts to construct yeast strains in which sucrose metabolism was  
27 dependent on *PvSUF1* led to slow sucrose uptake. Here, *PvSUF1*-dependent *S. cerevisiae* strains  
28 were evolved for faster growth. Of five independently evolved strains, two showed an  
29 approximately two-fold higher anaerobic growth rate on sucrose than the parental strain ( $\mu =$   
30  $0.19 \text{ h}^{-1}$  and  $\mu = 0.08 \text{ h}^{-1}$ , respectively). All five mutants displayed sucrose-induced proton uptake  
31 ( $13\text{-}50 \mu\text{mol H}^+ (\text{g biomass})^{-1} \text{ min}^{-1}$ ). Their ATP yield from sucrose dissimilation, as estimated  
32 from biomass yields in anaerobic chemostat cultures, was the same as that of a congeneric strain  
33 expressing the native sucrose symporter Mal11p. Four out of six observed amino acid  
34 substitutions encoded by evolved *PvSUF1* alleles removed or introduced a cysteine residue and  
35 may be involved in transporter folding and/or oligomerization. Expression of one of the evolved  
36 *PvSUF1* alleles (*PvSUF1*<sup>I209F C265F G326C</sup>) in an unevolved strain enabled it to grow on sucrose at the  
37 same rate ( $0.19 \text{ h}^{-1}$ ) as the corresponding evolved strain. This study shows how laboratory  
38 evolution can be used to improve sucrose uptake via heterologous plant transporters; sheds  
39 light into the importance of cysteine residues for efficient plant transporter expression in yeast  
40 and warrant reinvestigation of *PvSuf1*'s mechanism in a plant context.

41

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

## 44 INTRODUCTION

45 Sucrose ( $\alpha$ -D-glucopyranosyl-(1 $\leftrightarrow$ 2)- $\beta$ -D-fructofuranoside) is an intensively used carbon source  
46 in microbial biotechnology (Maiorella, Blanch, & Wilke, 1984; Marques, Raghavendran, Stambuk,  
47 & Gombert, 2016; Peters, Rose, & Moser, 2010) and plays a vital role as a carbon and energy  
48 carrier in plants (Salerno & Curatti, 2003). In plants, sucrose exits source cells via  
49 plasmodesmata as well as via membrane transport mediated by sucrose uniporters (Doidy et al.,  
50 2012).

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

58 Inspired by previous studies on free-energy conservation by *S. cerevisiae* during maltose  
59 consumption (de Kok et al., 2011), Marques et al. (2018) replaced yeast invertase by an  
60 intracellular sucrose phosphorylase from the bacterium *Leuconostoc mesenteroides*. Sucrose  
61 phosphorylase converts sucrose and inorganic phosphate into fructose and glucose-1-phosphate  
62 (glucose-1P) (Weimberg & Doudoroff, 1954), which can subsequently be isomerised to glucose-  
63 6P via the *S. cerevisiae* phosphoglucomutase (Pgm2). By circumventing the ATP-requiring  
64 hexokinase reaction for one of the monomers, this phosphorylase pathway saves one mole of  
65 ATP per mole of sucrose consumed. However, since phosphorylase takes place in the cytosol  
66 and uptake of sucrose in yeast involves proton symport via  $\alpha$ -glucoside transporters (e.g. Mal11,  
67 Mal21, Mal31, Mph2, Mph3) (Badotti et al., 2008; Stambuk et al., 1999), protons have to be  
68 exported back to the extracellular space via plasma membrane H<sup>+</sup>-ATPase (Pma1), which has a  
69 H<sup>+</sup>/ATP stoichiometry of 1:1 (Van Leeuwen, Weusthuis, Postma, Van den Broek, & Van Dijken,  
70 1992). For this reason, the overall free-energy conservation in the engineered 'phosphorolytic'

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

80 Many studies have demonstrated functional expression of heterologous transporters in *S.*  
81 *cerevisiae* (He, Wang, & Yan, 2014; Kim, Lee, Galazka, Cate, & Jin, 2014; Lin et al., 2014; Ton &  
82 Rao, 2004). However, efficient transporter sorting, folding and stability in yeast cells can be a  
83 major challenge (Froissard et al., 2006; Hernández, 2005). Therefore, functional characteristics  
84 of plant transporters expressed in *S. cerevisiae* cannot always be derived from *in planta* results  
85 (Bassham & Raikhel, 2000). Few previous studies have investigated the expression of plant  
86 sucrose uniporters in yeast (Chen, 2014; Lin et al., 2014; Zhou, Grof, & Patrick, 2014; Zhou, Qu,  
87 Dibley, Offler, & Patrick, 2007). Marques et al. (2018) expressed 5 candidate genes for sucrose  
88 uniporters in *S. cerevisiae*, of which only one, encoding the *Phaseolus vulgaris* Sucrose Facilitator  
89 1 (*PvSUF1*), supported growth of a strain (after one week time) in which extracellular invertase  
90 and sucrose symport have been eliminated. However, the anaerobic specific growth rate on  
91 sucrose of this strain was only 0.05 h<sup>-1</sup>, which was six-fold lower than that of the reference strain  
92 CEN.PK113-7D. Indeed, rates of uptake of radiolabelled sucrose by the *PvSUF1*-expressing strain  
93 were close to the detection limit. While some YPet-tagged *PvSuf1* was found at the yeast plasma  
94 membrane, it also accumulated in intracellular compartments, suggesting poor intracellular  
95 targeting and/or high turnover of heterologously expressed *PvSuf1* (Marques et al., 2018).

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

98 genetic factors involved in optimal functional expression of *PvSUF1* in this yeast. To this end, we  
99 used laboratory evolution to select for *PvSuf1*-dependent *S. cerevisiae* strains with improved  
100 sucrose-uptake kinetics and analysed causal mutations for improved sucrose consumption by  
101 evolved strains. To study the energy coupling of sucrose transport by evolved and unevolved  
102 *PvSuf1* variants, we analysed sucrose-induced proton-uptake by reference and evolved strains  
103 and measured biomass yields of yeast strains expressing different *PvSuf1* variants in anaerobic,  
104 sucrose-limited chemostat cultures.

105

## 106 **MATERIALS AND METHODS**

107

### 108 **Microbial strains and cultivation medium**

109 The *S. cerevisiae* strains used in this study (**Table 1, Figure 1**) share the CEN.PK genetic  
110 background (Entian & Kötter, 2007; Nijkamp et al., 2012). Cultures were grown in an Innova  
111 incubator shaker (Eppendorf, Hamburg, Germany) at 200 rpm, 30 °C, in 500 mL shake flasks  
112 containing 100 mL of either yeast-peptone-dextrose medium (YPD) or synthetic medium  
113 (Verduyn, Postma, Scheffers, & van Dijken, 1992) with 20 g/L glucose as the carbon source  
114 (SMD). Frozen stock cultures were prepared by adding glycerol (30 % v/v final concentration)  
115 to exponentially growing cells, followed by aseptic freezing and storage of 1 mL aliquots at -80  
116 °C.

117

### 118 **Molecular biology techniques**

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

125 (Thermo Fisher Scientific) in Tris-acetate-EDTA buffer (Thermo Fisher Scientific). DNA  
126 fragments were excised from the gels and purified by gel purification kit (D2004, Zymo  
127 Research). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich)  
128 according to the supplier's manual and from *S. cerevisiae* using Zymoprep Yeast Plasmid  
129 Miniprep II kit (Zymo Research). Restriction analysis was performed using FastDigest enzymes  
130 (Thermo Fisher Scientific) according to the manufacturer's manual. *E. coli* DH5 $\alpha$  cells (18258-  
131 012, Thermo Fisher Scientific) were transformed via electroporation using a Gene Pulser Xcell  
132 Electroporation System (Bio-Rad), following the manufacturers protocol.

133

### 134 **Sanger and whole-genome sequencing**

135 Genome-integrated and episomal expression cassettes present in the evolved strains (IMS644,  
136 IMS646, IMS647, IMS648 and IMS649) were Sanger sequenced at BaseClear BV (Leiden, The  
137 Netherlands). Primers 6018&7822 (**Table S1**) were used to amplify the *SPase*-expression  
138 cassette for sequencing. Similarly, primers 5606&7827 were used to amplify the *PvSUF1* allele of  
139 strain IMS648, before Sanger sequencing. Plasmids expressing the *PGM2* and *PvSUF1* genes were  
140 extracted from yeast using Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research) and  
141 transformed into *E. coli* (DH5 $\alpha$  cells, 18258-012, Thermo Fisher Scientific) via electroporation  
142 using a Gene Pulser Xcell Electroporation System (Bio-Rad) for propagation. After extraction  
143 from *E. coli* using the Sigma GenElute Plasmid kit (Sigma-Aldrich), plasmids were used as a  
144 template to sequence the *PGM2* and *PvSUF1* cassettes. Genes were sent for Sanger sequencing  
145 using the primers listed in **Table S1** resulting in a two times coverage of each base pair. The  
146 promoter and terminator regions sequenced were: 420 bp upstream and 280 bp downstream of  
147 the *PvSUF1* ORF, 500 bp upstream and 170 bp downstream of the *PGM2* ORF and 670 bp  
148 upstream and 370 bp downstream of the *LmSPase* ORF. Genomic DNA for whole-genome  
149 sequencing was extracted using the Qiagen 100/G kit following the manufacturer's protocol  
150 (Qiagen, Hilden, Germany), from shake-flask cultures grown on SMD. Whole-genome sequencing  
151 was performed by Novogene (HK) Company Ltd (Hong Kong, China). A PCR-free insert library of

152 350-bp genomic fragments was created and sequenced paired end (150-bp reads). A minimum  
153 data quantity of 2600 MB was generated per strain, representing a minimum 216-fold coverage.  
154 Data analysis was performed by mapping the sequence reads to the CEN.PK113-7D reference  
155 (Salazar et al., 2017) using the Burrows-Wheeler alignment (BWA) tool (Li & Durbin, 2009) and  
156 processed with Pilon (Walker et al., 2014). The sequencing data of the parental strain IMZ630  
157 and of the evolved isolates (IMS644, IMS646, IMS647, IMS648 and IMS649) were deposited at  
158 NCBI under the BioProject ID: PRJNA471800.

159

### 160 **Plasmid construction**

161 Plasmid assembly was performed by *in vivo* homologous recombination in *S. cerevisiae*  
162 transformed according to Gietz and Woods (2002) using 0.5 - 1.0 µg of DNA. Construction of the  
163 "empty" vector plasmid pUDE690 (**Table 2**) was done by amplifying pUDE486 in two parts,  
164 leaving out the *PvSUF1* ORF. Both parts were amplified using primers 11846 & 5975 and 5974 &  
165 11847 (**Table S1**). The ends of the amplified fragments shared 60 bp homology regions and  
166 were joined *in vivo* (Kuijpers et al., 2013) by transforming both parts in strain IMZ630 (**Table 1**).  
167 Plasmids pUDE544, pUDE546, pUDE547, pUDE559 and pUDE560 were extracted from sucrose-  
168 evolved strains IMS644, IMS646, IMS647, IMS648 and IMS649, respectively, and transformed  
169 into *E. coli* DH5α cells for plasmid multiplication and storage (**Table 1, Table 2**). *PvSUF1*-  
170 expression cassettes from pUDE413 and pUDE486 were replaced by *PvSUF1*-expression  
171 cassettes containing mutations in the *PvSUF1* ORF. For this purpose, pUDE413 or pUDE486  
172 backbone was amplified using primers 9041&5975 and assembled *in vivo* with *PvSUF1*-  
173 expression cassette amplified from pUDE545 or pUDE546 using primers 2889&10307 in strain  
174 IMZ630, resulting in plasmids pUDE565 and pUDE566, respectively, when pUDE413-backbone  
175 was used, and plasmids pUDE567 and pUDE568, respectively, when backbone from pUDE486  
176 was used (**Table 2**). pUDE691 was constructed in the same way as pUDE567 and pUDE568 with  
177 the exception that, for construction of this plasmid, the *PvSUF1*-expression cassette was  
178 amplified from pUDE560 (**Table 2**).



179

**180 Strain construction**

181 Strain IMZ730 was constructed by transforming pUDE690 into IMZ630 (**Figure 1**). Plasmids  
182 present in the evolved strains, IMS644, IMS646, IMS647, IMS648 and IMS649, were removed by  
183 overnight cultivation on YPD medium followed by selection on SMD agar plates supplemented  
184 with 0.15 g/L uracil and 1 g/L 5'-fluoroorotic acid (Boeke, La Croute, & Fink, 1984), resulting in  
185 strains IMS652, IMS653, IMS654, IMS655 and IMS656, respectively (**Figure 1**). Expression  
186 cassettes (*TEF1p-PvSUF1-CYC1t*) were extracted from the evolved strains, cloned into a 2- $\mu$ m  
187 plasmid (with and without *PGM2*) resulting in plasmids pUDE565, pUDE566, pUDE567,  
188 pUDE568 and pUDE691, which were subsequently transformed into an unevolved strain  
189 background (IMZ630), resulting in strains IMZ712, IMZ713, IMZ714, IMZ715 and IMZ729,  
190 respectively. Similarly, the *PvSUF1*-containing plasmids extracted from the evolved strains  
191 (pUDE545, pUDE546 and pUDE560) were transformed into an evolved background, IMS656  
192 (obtained after plasmid removal from strain IMS649), resulting in strains IMZ724, IMZ725 and  
193 IMZ727, respectively (**Figure 1**).

194

**195 Cultivation conditions**

196 Shake flask cultivations were carried out in 500 mL flasks containing 100 mL synthetic medium  
197 with 20 g/L initial sucrose (SMS) (if not stated, sugar concentration in SMD or SMS was 20 g/L),  
198 in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) set at 200 rpm and 30 °C.  
199 For growth rate determinations, frozen stock cultures were first inoculated in a shake flask  
200 containing SMD. After reaching stationary phase, cultures were transferred to SMS (initial  
201  $OD_{660nm} \leq 0.2$ ) and incubated under an anaerobic atmosphere (5% H<sub>2</sub> 6% CO<sub>2</sub> and 89% N<sub>2</sub>) in a  
202 Bactron X anaerobic chamber (Shell Lab, Cornelius, OR) until exponential growth was observed.  
203 Inside the anaerobic chamber, exponentially growing cultures were then transferred to fresh  
204 SMS (initial  $OD_{660nm} = 0.2$ ) and samples were taken hourly until stationary phase was reached.  
205 Specific growth rates were calculated from at least five OD measurements evenly distributed

206 over the exponential growth phase. For anaerobic cultivations, synthetic medium was  
207 supplemented with 10 mg/L ergosterol and 420 mg/L Tween 80. Since stock solutions of these  
208 anaerobic growth factors were prepared with ethanol, the initial ethanol concentration in media  
209 for anaerobic growth was 0.67 g/L. Chemostat cultivations were performed in 1.5 L bioreactors  
210 (800 rpm, 30°C) (Applikon, Delft, The Netherlands) with 1 L SMS supplemented with 0.15 g/L  
211 Antifoam C (Sigma-Aldrich), which was autoclaved separately (120 °C for 20 min) (Verduyn et  
212 al., 1992). The culture pH was maintained at 5.0 by automated addition of 2 M KOH. For aerobic  
213 cultivation, 500 mL min<sup>-1</sup> compressed air was sparged in the reactor. To maintain anaerobic  
214 conditions, the bioreactors were sparged with 500 mL N<sub>2</sub> min<sup>-1</sup> (<5 ppm O<sub>2</sub>) (also the medium  
215 vessels were sparged with N<sub>2</sub>) and equipped with Norprene tubing to minimize oxygen diffusion.  
216 After the batch phase, medium pumps were switched on, resulting in the continuous addition of  
217 SMS containing 25 g/L sucrose to the cultures. The working volume was kept constant at 1.0 L  
218 using an effluent pump controlled by an electric level sensor, resulting in a constant dilution  
219 rate. The exact working volume and medium flow rate were measured at the end of each  
220 experiment. Chemostat cultures were assumed to be in steady state when, after five volume  
221 changes, the biomass concentration and the CO<sub>2</sub> production rate varied by less than 4.5 % over  
222 at least another 2 volume changes.

223

## 224 **Laboratory evolution**

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

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

**260 Analytical methods**

261 Optical density of cultures at 660 nm was monitored using a Libra S11 spectrophotometer  
262 (Biochrom, Cambridge, United Kingdom). Culture dry weight measurements and rapid quenching  
263 of culture samples were carried out as described by Marques et al. (2018). HPLC analysis of the  
264 supernatant and residual sugar determination were performed as described previously (de Kok  
265 et al., 2011; Marques et al., 2017). Off-gas CO<sub>2</sub> concentrations were measured with an NGA 2000  
266 Rosemount gas analyser (Emerson, St. Louis, MO).

267

**268 Proton translocation assay**

269 Cells used in proton translocation assays were harvested from aerobic sucrose-limited  
270 chemostat cultures. The same culture conditions as described for anaerobic chemostats above  
271 were used, with the following changes: reactors were sparged with 500 mL min<sup>-1</sup> air, the sucrose  
272 concentration in the medium vessel was decreased to 7.5 g/L, the dilution rate was set at 0.03 h<sup>-1</sup>  
273 and Tween 80 and ergosterol were omitted from the medium. After 5 volume changes, when  
274 CO<sub>2</sub> concentrations in the off-gas were stable, cells were harvested by centrifugation at 5 000 x *g*  
275 for 5 min, at room temperature, washed with distilled water and resuspended in potassium  
276 phthalate buffer (1.25 mM, pH 5) to a final concentration of 12 g dry weight L<sup>-1</sup>. 5 mL of the cell  
277 suspension were incubated at 30 °C in a magnetic stirred vessel with a S220 SevenCompact™  
278 pH/Ion electrode attached (Mettler Toledo, Greifensee, Switzerland). After stabilization of the  
279 pH signal, 100 µL of a 1 mol L<sup>-1</sup> sugar (sucrose, maltose, glucose or fructose) solution was added  
280 (final concentration 20 mM) and changes in the pH were recorded using the LabX™ pH Software  
281 (Mettler Toledo, Greifensee, Switzerland). Calibration was performed by addition of 5 µL  
282 aliquots of 10 mM NaOH to the cell suspensions.

283

**284 RESULTS**

285

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

288 In the engineered *S. cerevisiae* strain IMZ636 (*malΔ mphΔ suc2Δ imaΔ sga1Δ LmSPase PvSUF1*),  
289 sucrose metabolism is strictly dependent on uptake via the plant transporter *PvSuf1* and  
290 subsequent intracellular cleavage by a bacterial sucrose phosphorylase. In anaerobic shake flask  
291 cultures, strain IMZ636 grew on SMS (synthetic medium with 20 g/L sucrose as sole carbon  
292 source) at a specific growth rate of 0.05 h<sup>-1</sup> (**Table 3**). This growth rate is much lower than that  
293 of the reference strain CEN.PK113-7D (0.29 h<sup>-1</sup>, **Table 3**). To select for faster growth on sucrose,  
294 strain IMZ636 was grown in anaerobic sequencing-batch bioreactors (SBR). In three  
295 independent evolution lines, the specific growth rate increased from 0.05 h<sup>-1</sup> to 0.10-0.17 h<sup>-1</sup>  
296 (estimated from off-gas CO<sub>2</sub> profiles) after 56-80 cycles (approximately 150 generations, **Figure**  
297 **2**). When no further increase in the growth rate was observed, four single colonies were isolated  
298 from each experiment and characterized in anaerobic shake-flask cultures on SMS. Differences in  
299 growth rate among the four colonies isolated from each evolution line indicate heterogeneity in  
300 the evolved population (results not shown). The fastest isolate of each evolution line displayed  
301 specific growth rates of 0.07 h<sup>-1</sup> (IMS644 from reactor "A"), 0.09 h<sup>-1</sup> (IMS646 from reactor "B")  
302 and 0.08 h<sup>-1</sup> (IMS647 from reactor "C") (**Table 3**). These growth rates were consistently higher  
303 than those of the unevolved IMZ636 strain, but differed slightly from those estimated from CO<sub>2</sub>  
304 profiles in the SBR experiments. These differences may have been caused by the different  
305 methods used to access growth rate (optical density measurements versus on-line CO<sub>2</sub> data;  
306 shake flasks versus bioreactors). Additionally, the shake-flask experiments were performed with  
307 single cell lines while the specific growth rates estimated from the SBR experiments represented  
308 growth of an evolving and probably heterogeneous population.

309 The anaerobic specific growth rates on sucrose of the evolved, IMZ636-derived strains were still  
310 3-4 fold lower than that of the congeneric reference strain CEN.PK113-7D (**Table 3**). Marques et  
311 al. (2018) showed that overexpression of phosphoglucomutase (*PGM2*) in a unevolved *PvSUF1*-  
312 *SPase*-expressing strain increased its growth rate from 0.05 to 0.07 h<sup>-1</sup> (IMZ696). To investigate

313 whether in the laboratory evolution experiments, specific growth rates were limited by  
314 phosphoglucosyltransferase levels, a new sequential batch cultivation evolution was initiated with a  
315 *PGM2*-expressing strain (IMZ696-*PvSUF1*, *SPase*, *PGM2*). In two independent evolution lines, the  
316 specific growth rate on sucrose increased from 0.07 to 0.15-0.20 h<sup>-1</sup> (calculated from the off-gas  
317 CO<sub>2</sub> concentration from each reactor) after 52 cycles (ca. 120 generations in reactor A and 190  
318 generations in reactor B, **Figure 3A** and **3B**). Since no further increase in growth rate was  
319 observed in this reactor's configuration, laboratory evolution was continued using accelerostat  
320 cultivation, with an initial dilution rate of 0.09 h<sup>-1</sup>. In accelerostats, the selective pressure is on  
321 growth rate but also on substrate affinity (Bracher et al., 2017). The feed rate was automatically  
322 increased or decreased based on on-line analysis of the CO<sub>2</sub> concentration in the off-gas. After  
323 20-30 days (approximately 130 generations), the dilution rate in the two reactors had increased  
324 to 0.25 h<sup>-1</sup> and 0.17 h<sup>-1</sup> (**Figures 3C** and **3D**), while the residual sucrose concentration had  
325 decreased from 8 g/L to approximately 2.5 g/L after accelerostat selection (results not shown).  
326 These dilution rates were close to the anaerobic specific growth rate on sucrose reported for a  
327 *LmSPase*-dependent strain expressing the native *MAL11* sucrose symporter (0.23 h<sup>-1</sup>, Marques et  
328 al. 2018). Single colony isolates from each reactor (IMS648 from reactor shown in **Figure 3A**  
329 and IMS649 from reactor shown in **Figure 3B**) exhibited specific growth rates of 0.19 h<sup>-1</sup> and  
330 0.23 h<sup>-1</sup>, respectively (**Table 3**). These specific growth rates were close to that of IMZ709  
331 (*MAL11*, *SPase*, *PGM2*), and almost three times higher than that of the unevolved parental strain  
332 IMZ696 (**Table 3**).

333

### 334 **Sucrose/H<sup>+</sup> symport activity of evolved *PvSuf1* transporters as revealed by proton-uptake** 335 **studies and biomass yields on sucrose**

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

340 *SPase, PGM2*) grown in aerobic, sucrose-limited chemostat cultures (**Figure 4**, data extracted  
341 from Marques et al. 2018). This measured uptake rate would in principle be more than sufficient  
342 to account for all sucrose uptake in the aerobic chemostat cultures. If we considered the residual  
343 sucrose concentration in the bioreactor negligible, a specific sucrose uptake rate of  $3.7 \pm 0.1$   
344  $\mu\text{mol sucrose (g biomass)}^{-1} \text{ min}^{-1}$  would be calculated. In replicate chemostat experiments with  
345 strain IMZ696, different mutations were found in *PvSUF1* (*PvSuf1*<sup>T302I</sup> in one culture and  
346 *PvSuf1*<sup>E308K V323F</sup> in the other). Since the frozen stock cultures from which the chemostats were  
347 inoculated did not contain mutations in *PvSUF1*, these mutations probably conferred a selective  
348 advantage during sucrose-limited chemostat cultivation (Marques et al. 2018).

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

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

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

370 The strains used in this study cleaved sucrose intracellularly via phosphorolysis. In such strains,  
371 expression of a sucrose/H<sup>+</sup> symporter should result in a net generation of 4 mol ATP per mol  
372 sucrose under anaerobic conditions. If sucrose uptake occurred by uniport, this ATP yield would  
373 change to 5 ATP/sucrose, a 25% increase (Marques et al., 2018). In sucrose-limited, anaerobic  
374 chemostat cultures, a 25% increase of the ATP yield from sucrose dissimilation should result in a  
375 25% increase in the biomass yield on sucrose (Verduyn et al., 1990). Two of the evolved strains  
376 (IMS646 from the evolution started with IMZ636 and IMS649 from the evolution started with  
377 IMZ696) were characterized in chemostat cultures and their biomass yields were compared to  
378 those displayed by reference strains (IMZ665 and IMZ709), which both expressed *MAL11*  
379 instead of *PvSUF1*. No differences in biomass yield were observed between a *MAL11* expressing  
380 strains and strains evolved with *PvSUF1*: the observed biomass yield of strain IMZ665 (*MAL11*,  
381 *SPase*) was  $0.086 \pm 0.002$  g (g glucose equivalent)<sup>-1</sup> while that of IMS646 (*PvSUF1*, *SPase*) was  
382  $0.082 \pm 0.004$  g (g glucose equivalent)<sup>-1</sup> (**Table 4** and **Table S2**). Similarly, for the strains  
383 expressing *PGM2* (IMZ709 and IMS649) the biomass yield was not higher in cultures of the  
384 evolved *PvSUF1*-expressing strain ( $0.087 \pm 0.000$  g (g glucose equivalent)<sup>-1</sup> for IMS649) than in  
385 cultures of the *MAL11*-expressing strain ( $0.091 \pm 0.006$  g (g glucose equivalent)<sup>-1</sup> for IMZ709)  
386 (**Table 4** and **Table S2**).

387

### 388 **Evolved strains contain mutations in *PvSUF1***

389 Non-conservative single-nucleotide mutations were detected in the *PvSUF1* open reading frames  
390 of all *PvSuf1*-dependent strains evolved for faster growth on sucrose (IMS644, IMS646, IMS647,  
391 IMS648 and IMS649; **Figure 5**). No mutations were found in the promoter (*TEF1*) or in the  
392 terminator (*CYC1*) regions of the *PvSUF1* expression cassettes. Strains IMS644 and IMS647,  
393 which were independently evolved from strain IMZ636 (*PvSUF1,SPase*), contained the same



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

413

#### 414 **Independently evolved strains in a sucrose-based medium show common whole-** 415 **chromosome and segmental aneuploidies**

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

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

427

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

429 To investigate whether the faster growth on sucrose observed after evolution of *PvSUF1*-  
 430 expressing strains could be exclusively attributed to the mutations found in *PvSUF1*, *PvSUF1*  
 431 expression cassettes (*TEF1p-PvSUF1-CYC1t*) were first isolated from each of the evolved strains  
 432 (IMS646, IMS647 and IMS649) and cloned into a 2- $\mu$ m plasmid. Two versions were constructed,  
 433 one carrying an additional *PGM2*-expressing cassette and another one without it. The resulting  
 434 plasmids were transformed into an unevolved background strain, IMZ630 (*malA mphA suc2A*  
 435 *imaA SPase*), resulting in strains IMZ712-715 and IMZ729 (**Table 6**). Strain IMZ712 (expressing  
 436 *PvSUF1*<sup>G326C</sup>) grew anaerobically on sucrose at 0.05 h<sup>-1</sup> (**Table 6**), which corresponds to the  
 437 specific growth rate displayed by the unevolved parental strain (IMZ636, **Table 3**). Co-  
 438 expression of *PGM2* did not lead to a higher growth rate (IMZ714,  $\mu = 0.05$  h<sup>-1</sup>, **Table 6**).  
 439 Overexpression of a *PvSUF1*<sup>YAAGSFSG-duplication</sup> variant that encoded the 8 amino-acid duplication  
 440 described above (from strain IMS647) in the unevolved background also, by itself, did not result  
 441 in an increased specific growth rate (IMZ713  $\mu = 0.05$  h<sup>-1</sup>) (**Table 6**). However, when this  
 442 mutation was combined with the overexpression of *PGM2* (IMZ715), a specific growth rate of  
 443 0.10 h<sup>-1</sup> was observed (**Table 6**). The *PvSUF1* allele derived from strain IMS649, the evolved  
 444 strain that grew at 0.20 h<sup>-1</sup> (**Table 3**), supported high specific growth rates upon introduction in  
 445 a unevolved background when combined with *PGM2* overexpression (strain IMZ729,  $\mu = 0.17 \pm$   
 446 0.02 h<sup>-1</sup>) (**Table 6**).

447 To investigate the possible impact of mutations outside *PvSUF1*, the *PvSUF1* expression  
448 plasmid was cured from the fastest growing evolved strain IMS649. The resulting strain  
449 (IMS656) was then directly transformed with the vectors extracted from the evolved strains  
450 carrying *PvSUF1* variants (*PvSUF1*<sup>G326C</sup>, *PvSUF1*<sup>YAAGSFSG-duplication</sup> and *PvSUF1*<sup>I209F C265F G326C</sup>) yielding  
451 strains IMZ724, IMZ725 and IMZ727, respectively. The growth rates of these strains (0.07 h<sup>-1</sup>,  
452 0.06 h<sup>-1</sup> and 0.17 h<sup>-1</sup>, respectively) were similar to that of the strains expressing evolved *PvSUF1*  
453 in an unevolved background (**Table 6**). This result shows that the improved growth of strain  
454 IMS649 was predominantly due to mutations in *PvSUF1* and that other chromosomal alterations  
455 had at most a small impact on the strain's specific growth rate on sucrose (**Table 6**).

456

## 457 DISCUSSION

458 Optimal expression of heterologous transporters in yeast can be hampered by protein  
459 misfolding, incorrect sorting, cell toxicity due to protein accumulation in intracellular  
460 compartments (unfolded protein response - UPR), rapid endocytosis and turnover, among other  
461 phenomena (Bassham & Raikhel, 2000; Froissard et al., 2006; Hernández, 2005; Nielsen, 2013).  
462 In this study, genetic factors involved in the efficient expression of a putative plant sucrose  
463 uniporter (*PvSuf1*) in *S. cerevisiae* were identified by a combination of laboratory evolution,  
464 whole genome sequencing, reverse engineering of mutations observed in evolved strains and  
465 physiological analysis of evolved and reverse engineered strains.

466 Laboratory evolution of yeast dependent on sucrose uptake via *PvSuf1* resulted in faster  
467 growing strains – when compared to the parental ones – which contained mutations in the  
468 *PvSUF1* allele (**Figure 5**). Besides faster growth, the mutations may also be responsible for  
469 lowering the residual sucrose concentration in the accelerostat reactor (from 8 to 2.5 g/L, see  
470 Results section). Chromosomal duplications were also found in the genome of the evolved  
471 strains (**Table 5, Figure S2**) affecting genes that encode proteins involved in transporter  
472 sorting, ubiquitination and degradation: *COS10*, *SEC12* and *SIS1*, which are present on  
473 chromosome 14 (Luke, Sutton, & Arndt, 1991; Macdonald et al., 2015; Nakano, Brada, &

474 Schekman, 1988); *CUR1* and *SEC23* from chromosome 16 (Alberti, 2012) and *UBC7* found on  
475 chromosome 13 (Hiller, Finger, Schweiger, & Wolf, 1996). However, these genomic alterations  
476 are unlikely to be the main cause of the improved specific growth rate of the evolved strains  
477 since reverse engineering of a mutated version of *PvSUF1* in unevolved *S. cerevisiae* sufficed to  
478 generate a strain that grew as fast as the fastest-growing evolved strain (**Table 6**). Among the  
479 mutations found in *PvSUF1*, those involving cysteine residues predominated (*PvSUF1*<sup>G326C</sup>,  
480 *PvSUF1*<sup>G457D Y128C C228G</sup>, *PvSUF1*<sup>I209F C265F G326C</sup>; **Figure 5**). Correct formation of disulfide bonds has  
481 previously been reported to be important for inter- and intramolecular interactions of SUT  
482 (sucrose transporter) proteins and sucrose uptake activity (Krügel et al., 2008, 2012). *PvSuf1*  
483 contains only 3 out of the 4 cysteine residues conserved in all other plant sucrose transporters  
484 (**Figure 5**) (Lemoine, 2000). Since the *PvSUF1*<sup>G326C</sup> mutation was found in two independently  
485 evolved strains (IMS649 and IMS646), presence of a cysteine in position 326 of *PvSuf1* may be  
486 important for protein folding and oligomerization in yeast, but further research is clearly needed  
487 to test this hypothesis.

488 Sugar-proton symport assays showed that all *PvSUF1*-expressing *S. cerevisiae* strains tested  
489 (including those that were not subjected to laboratory evolution) displayed at least some  
490 sucrose/H<sup>+</sup> symport activity, which was high enough to explain their observed rates of sucrose  
491 uptake (**Figure 4**). While, based on heterologous expression, we cannot draw definitive  
492 conclusions on energy coupling of wild type *PvSuf1*-mediated sucrose transport, our results  
493 warrant reinvestigation of *PvSuf1*'s mechanism in a plant context.

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

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

503

504

505

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514

515 **Conflict of interest.** None declared

516

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- 673

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

Strain	Relevant genotype	Parental strain	Source
CEN.PK113-7D	MATa <i>URA3 LEU2 MAL2-8<sup>c</sup> SUC2</i>		Entian and Kötter, 2007
IMZ630	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase</i>		Marques et al. 2018
IMZ730	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE690 (URA3 PGM2)</i>	IMZ630	This study
IMZ636	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE413 (URA3 PvSUF1)</i>	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 <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE486 (URA3 PvSUF1 PGM2)</i>	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 <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase Plasmid cured</i>	IMS644	This study
IMS653	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase Plasmid cured</i>	IMS646	This study
IMS654	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase Plasmid cured</i>	IMS647	This study
IMS655	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase PvSUF1::PGM2* Plasmid cured</i>	IMS648	This study
IMS656	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase Plasmid cured</i>	IMS649	This study
IMZ712	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE565 (URA3 PvSUF1<sup>G326C</sup>)</i>	IMZ630	This study
IMZ713	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE566 (URA3 PvSUF1<sup>YAAGSFSG-duplication</sup>)</i>	IMZ630	This study
IMZ714	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE567 (URA3 PvSUF1<sup>G326C::PGM2</sup>)</i>	IMZ630	This study
IMZ715	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE568 (URA3 PvSUF1<sup>YAAGSFSG-duplication::PGM2</sup>)</i>	IMZ630	This study
IMZ729	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE691 (URA3 PvSUF1<sup>I209F-C265F-G326C</sup> PGM2)</i>	IMZ630	This study
IMZ724	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE545 (URA3 PvSUF1<sup>G326C</sup>)</i>	IMS656	This study
IMZ725	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE546 (URA3 PvSUF1<sup>YAAGSFSG-duplication</sup>)</i>	IMS656	This study
IMZ727	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE560 (URA3 PvSUF1<sup>I209F-C265F-G326C</sup> PGM2)</i>	IMS656	This study
IMZ665	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE432 (URA3 MAL11)</i>		Marques et al. 2018
IMZ709	MATa <i>ura3-52 LEU2 MAL2-8<sup>c</sup> malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE496 (URA3 MAL11 PGM2)</i>		Marques et al. 2018

675 \*"*PvSUF1::PGM2*" expression cassette migrated from the plasmid to a chromosome. The exact site of integration was not  
676 investigated in this work.  
677  
678

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

<b>Name</b>	<b>Relevant characteristics</b>	<b>Origin</b>
pUDE413	<i>2μ URA3 pTEF1-PvSUF1-tCYC1</i>	Marques et al. 2018
pUDE486	<i>2μ URA3 pTEF1-PvSUF1-tCYC1 pTPI1-PGM2-tTEF1</i>	Marques et al. 2018
pUDE690	<i>2μ URA3 pTPI1-PGM2-tTEF1</i>	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	<i>2μ URA3 pTPI1-PvSUF1<sup>G326C</sup>-tCYC1</i>	This study
pUDE566	<i>2μ URA3 pTPI1-PvSUF1<sup>YAAGSFSG-duplication</sup>-tCYC1</i>	This study
pUDE567	<i>2μ URA3 pTPI1-PvSUF1<sup>G326C</sup>-tCYC1 pTPI1-PGM2-tTEF1</i>	This study
pUDE568	<i>2μ URA3 pTPI1-PvSUF1<sup>YAAGSFSG-duplication</sup>-tCYC1 pTPI1-PGM2-tTEF1t</i>	This study
pUDE691	<i>2μ URA3 pTPI1-PvSUF1<sup>I217F, C265F, G326C</sup>-tCYC1 pTPI1-PGM2-tTEF1</i>	This study

680

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 2 $\mu$ -plasmid with or without *PGM2*.

Strain	Relevant characteristics	Specific growth rate (h <sup>-1</sup> )
CEN.PK113-7D	<i>SUC2</i>	0.29 ± 0.00
IMZ636	<i>SPase, PvSUF1</i>	0.05 ± 0.01
IMS644	<i>SPase, PvSUF1</i> , evolved "A"	0.07 ± 0.00
IMS646	<i>SPase, PvSUF1</i> , evolved "B"	0.09 ± 0.01
IMS647	<i>SPase, PvSUF1</i> , evolved "C"	0.08 ± 0.01
IMZ696	<i>SPase, PvSUF1-PGM2</i> , parental strain	0.07 ± 0.01
IMS648	<i>SPase, PvSUF1*-PGM2</i> , evolved "A"	0.18 ± 0.01
IMS649	<i>SPase, PvSUF1-PGM2</i> , 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<sub>2</sub>/min). A dilution  
 690 rate of 0.07 h<sup>-1</sup> was used for strains IMZ665 (*MAL11*, *SPase*) and IMS646 (*PvSUF1*, *SPase*), while  
 691 0.15 h<sup>-1</sup> 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)
<b>Relevant genotype</b>	<i>MAL11</i> <i>LmSPase</i>	<i>PvSUF1</i> <i>LmSPase</i>	<i>MAL11</i> <i>LmSPase</i> <i>PGM2</i>	<i>PvSUF1</i> <i>LmSPase</i> <i>PGM2</i>
Actual dilution rate (h <sup>-1</sup> )	0.070 ± 0.000	0.071 ± 0.001	0.147 ± 0.001	0.152 ± 0.001
Biomass yield (g g glucose equivalent <sup>-1</sup> )	0.086 ± 0.002	0.082 ± 0.004	0.091 ± 0.006	0.087 ± 0.000
q <sub>sucrose</sub> (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

697 **Table 5:** Summary of whole-chromosome and segmental aneuploidies found in *PvSUF1*-  
 698 expressing strains evolved on sucrose-based medium relative to the unevolved reference strain  
 699 IMZ630. "Chr": chromosome. "+": presence of copy number variation. Chromosome positions are  
 700 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				+	+

701

702

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	<i>PvSUF1</i> from evolved strain	Relevant characteristic	Specific growth rate (h <sup>-1</sup> )
IMZ712	IMZ630	IMS646	<i>SPase, PvSUF1</i> <sup>G326C</sup>	0.05 ± 0.01
IMZ713	IMZ630	IMS647	<i>SPase, PvSUF1</i> <sup>YAAGSFSG-duplication</sup>	0.05 ± 0.01
IMZ714	IMZ630	IMS646	<i>SPase, PvSUF1</i> <sup>G326C</sup> <i>PGM2</i>	0.05 ± 0.01
IMZ715	IMZ630	IMS647	<i>SPase, PvSUF1</i> <sup>YAAGSFSG-duplication</sup> <i>PGM2</i>	0.10 ± 0.01
IMZ729	IMZ630	IMS649	<i>SPase, PvSUF1</i> <sup>I209F C265F G326C</sup> <i>PGM2</i>	0.17 ± 0.02
IMZ724	IMS656	IMS646	<i>SPase, PvSUF1</i> <sup>G326C</sup>	0.07 ± 0.01
IMZ725	IMS656	IMS647	<i>SPase, PvSUF1</i> <sup>YAAGSFSG-duplication</sup>	0.06 ± 0.01
IMZ727	IMS656	IMS649	<i>SPase, PvSUF1</i> <sup>I209F C265F G326C</sup> <i>PGM2</i>	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<sub>2</sub> gas. Evolution was  
 717 conducted independently in triplicate (panels **A**, **B** and **C**). Specific growth rates were estimated  
 718 from the corresponding CO<sub>2</sub> profiles (not shown here) in the off-gas. 100 mL of SMS were used  
 719 (pH 5.0, 30 °C, 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<sub>2</sub> 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<sup>-1</sup> dilution rate). Feed pump speed  
 728 (black line) was increased automatically based on the CO<sub>2</sub> concentration in the off-gas (blue  
 729 line). A maximum pump speed corresponding to 0.25 h<sup>-1</sup> dilution rate was reached in one  
 730 reactor (**C**) and of 0.17 h<sup>-1</sup> 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 °C, 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 ( $D = 0.03 \text{ h}^{-1}$ ,  
 739 30 °C, pH 5.0), washed and immediately tested for proton uptake upon addition of sucrose  
 740 (black bar), maltose (grey bar), fructose (\*: H<sup>+</sup> uptake not detected) or glucose (white bar, H<sup>+</sup>



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

746

747 **Figure 5:** Mutations found in *PvSUF1* after laboratory evolution on sucrose-based medium.  
748 Mutated amino acids are highlighted in red and the corresponding strain numbers are indicated.  
749 "AA": amino acids. Arrows indicate the location of four conserved cysteine residues in plant  
750 sucrose transporters. The cysteine at loop 2/3 is substituted by a serine in the wild-type *PvSuf1*  
751 protein. Membrane insertion of *PvSuf1* was predicted with the *Protter* algorithm (Omasits et al.,  
752 2014).

753

754 **Figure 6:** Model of the tertiary structure of wild-type *PvSuf1*. Extracellular loops are shown at  
755 the top and intracellular loops at the bottom of the figure "A". View from the extracellular space  
756 is shown in "B". The 8 amino-acid region in loop 5/6 that was duplicated in strains IMS644 and  
757 IMS647 is shown in red. In the same loop, isoleucine 209, which was mutated to phenylalanine  
758 (I209F) in strain IMS649 is highlighted in yellow. Images were prepared with PyMOL™ (version  
759 1.7.4.5 Educational Product, Schrodinger, LLC).

760

761