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Elimination of sucrose transport and hydrolysis in *Saccharomyces cerevisiae*: a platform strain for engineering sucrose metabolism

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One sentence summary: A yeast strain without functional sucrose transport and hydrolysis was constructed and is now ready to be used for metabolic engineering strategies and for fundamental studies into sucrose metabolism.

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\textbf{ABSTRACT}

Many relevant options to improve efficacy and kinetics of sucrose metabolism in *Saccharomyces cerevisiae* and, thereby, the economics of sucrose-based processes remain to be investigated. An essential first step is to identify all native sucrose-hydrolysing enzymes and sucrose transporters in this yeast, including those that can be activated by suppressor mutations in sucrose-negative strains. A strain in which all known sucrose-transporter genes (*MAL11*, *MAL21*, *MAL31*, *MPH2*, *MPH3*) were deleted did not grow on sucrose after 2 months of incubation. In contrast, a strain with deletions in genes encoding sucrose-hydrolysing enzymes (*SUC2*, *MAL12*, *MAL22*, *MAL32*) still grew on sucrose. Its specific growth rate increased from 0.08 to 0.25 h\textsuperscript{−1} after sequential batch cultivation. This increase was accompanied by a 3-fold increase of in vitro sucrose-hydrolysis and isomaltase activities, as well as by a 3- to 5-fold upregulation of the isomaltase-encoding genes *IMA1* and *IMA5*. One-step Cas9-mediated deletion of all isomaltase-encoding genes (*IMA1*-5) completely abolished sucrose hydrolysis. Even after 2 months of incubation, the resulting strain did not grow on sucrose. This sucrose-negative strain can be used as a platform to test metabolic engineering strategies and for fundamental studies into sucrose hydrolysis or transport.

Keywords: disaccharide; isomaltase; laboratory evolution; reverse engineering; multiple gene deletion; real-time PCR

\textbf{INTRODUCTION}

Sucrose is a cheap substrate for industrial fermentation that is extensively used for ethanol production (Maiorella, Blanch and Wilke 1984; Della-Bianca et al. 2013; Gombert and van Maris 2015) and can be used for a wide range of other yeast-based industrial processes (Marques et al. 2016; Meadows et al. 2016). The main
sources of sucrose are sugar cane in the tropics and sugar beet in temperate regions. Extraction of sucrose from feedstocks is inexpensive and no enzymatic pre-treatment is necessary prior to microbial fermentation (Maiorella, Blanch and Wilke 1984; Marques et al. 2016). Furthermore, the use of sucrose for industrial biotechnology does not have to compete with food production. For example, in the Brazilian ethanol industry, food and biofuel production can coexist (Mitchell 2008). Therefore, diversifying the range of products that can be produced from sucrose fermentation will have a positive impact on sustainable industrial production of fuels and chemicals (Meadows et al. 2016).

Sucrose-based ethanol production employs bakers’ yeast (Saccharomyces cerevisiae), which efficiently consumes sugars and can withstand industrially relevant stresses such as high ethanol and acid concentrations (Della-Bianca et al. 2014). To convert sucrose into hexoses, S. cerevisiae secretes invertase (β-fructosidase, sucrose hydrolase; Suc) (Winge and Roberts 1952; Hawthorne 1958). Invertase hydrolyses sucrose into the monomers, glucose and fructose, which subsequently enter the cell through facilitated diffusion via hexose transporters and are metabolised via the classical Embden-Meyerhof-Parnas glycolytic pathway (Lagunas 1993). Alternatively, sucrose can be imported by a proton symporter (Mal11, formerly known as Agt1) and hydrolysed in the cytosol (Stambuk et al. 1999). Cytosolically localised invertase and maltases (Malx2) then hydrolyse sucrose intracellularly. In vitro studies have shown that isoamylases can also hydrolyse sucrose, but their contribution to in vivo sucrose metabolism remains to be addressed (Carlson and Botstein 1982; Stambuk et al. 1999; Badotti et al. 2008; Brown, Murray and Verstrepen 2010; Voordecker et al. 2012; Deng et al. 2014). Even closely related yeast strains exhibit significant diversity in the identity and number of representatives from each of the three disaccharide hydrolase-encoding gene families (SUC, MALx2, IMA) (Carlson and Botstein 1983; Brown, Murray and Verstrepen 2010). For example, the genome of the haploid laboratory strain S. cerevisiae CEN.PK113-7D contains five different IMA genes on subtelomeric regions: IMA1 (three copies: CHRIII, CHRVII, CHRXI), IMA2 (CHRXV), IMA3 (CHRIX), IMA4 (CHRX) and IMAS (CHRX) (Teste, Françoys and Parrou 2010; Nijkamp et al. 2012), while the related strain CEN.PK102-3A, which was used in this study, does not contain the IMA1 copy at CHRXI (de Kok et al. 2011).

Detailed complete knowledge of sucrose transport and hydrolysis is important for both industry and academia. Functional analysis of eukaryotic sucrose transporters and hydrolytic or phosphoroclastic sucrose-cleaving enzymes is often done in S. cerevisiae. An example is the development of a functional screening system for plant sucrose exporters (Zhou, Grof and Verduyn 2011). An example is the development of a functional screening system for plant sucrose exporters (Zhou, Grof and Verduyn 2011). An example is the development of a functional screening system for plant sucrose exporters (Zhou, Grof and Verduyn 2011).

In metabolic engineering, incomplete knowledge of the targeted pathway can delay strain construction due to the activity of competing pathways or activation of such pathways by suppressor mutations. Although mutants that are unable to grow on sucrose have been reported (Carlson, Osmond and Botstein 1981), it has not yet been investigated whether these strains can regain the ability to grow on sucrose after laboratory evolution (Marques et al. 2016), for instance through recruitment of any of the numerous glycoside hydrolases in S. cerevisiae (Yamamoto et al. 2004; Naumoff and Naumov 2010; Naumoff 2011). To address this question, S. cerevisiae strains in which either the genes encoding known sucrose transporters or those encoding disaccharide-hydrolysing enzymes were inactivated were subjected to laboratory evolution and mutations underlying the acquired phenotypes were identified by whole-genome resequencing.

MATERIAL AND METHODS

Strains and maintenance

Saccharomyces cerevisiae strains used in this work (Table 1) belong to the CEN.PK family (Entian and Köttler 2007; Nijkamp et al. 2012). To prepare stock cultures, yeast strains were grown in 50 mL shake flasks containing 10 mL of either yeast-peptone-dextrose (YPD) medium or, for strains carrying plasmids with auxotrophic marker genes, synthetic medium (SM). YPD contained 10 g L\(^{-1}\) Bacto yeast extract, 20 g L\(^{-1}\) Bacto peptone and 20 g L\(^{-1}\) glucose in demineralised water. SM, supplemented with vitamins, trace elements and 20 g L\(^{-1}\) glucose, was prepared according to Verduyn et al. (1992). Glycerol was added to growing cultures (final concentration 30% v/v) and 1 mL aliquots were stored at –80°C.

Molecular biology techniques

Diagnostic PCR was performed using DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification for cloning and sequencing purposes was performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Both enzymes were used according to the manufacturer’s instructions. Primers and oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNA purification from PCR reaction mixtures was done using GenElute PCR Clean-up Kit (Sigma-Aldrich). Separation of DNA fragments was performed in 1% (w/v) agarose gel (Thermo Fisher Scientific) in TAE buffer (40 mM Tris-acetate pH 8.0 and 1 mM EDTA). DNA fragments were purified from gels using the Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA, USA). Plasmid assembly was done with the Gibson Assembly Master Mix (New England Biolabs, Beverly, MA) according to the manufacturer’s protocol. Restriction endonucleases (Thermo Fisher Scientific) and T4 DNA ligase (Promega Corporation, Madison, WI, USA) were used according to the manufacturer’s instructions. Plasmids were isolated from Escherichia coli using GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich) according to the provided protocol. Yeast genomic DNA was extracted using YeaStar Genomic kit (Zymo Research).
Table 1. Strains used in this study. The abbreviation malΔ indicates mal1-11-mal12::loxP mal21-22::loxP mal31-32::loxP and mphΔ indicates mph2/3::loxP mph2/3::loxP-hphNT1-loxP.

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<th>Parental strain</th>
<th>Origin</th>
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<td>CEN.PK113-7D</td>
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<td>CEN.PK102-3A</td>
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<td></td>
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Table 2. Plasmids used in this study.

<table>
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<th>Name</th>
<th>Relevant characteristics</th>
<th>Origin</th>
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<tr>
<td>pUG6</td>
<td>loxP-KanMX4-foXP</td>
<td>Gueldener et al. (2002)</td>
</tr>
<tr>
<td>p426-GPD</td>
<td>2μm URA3 Pfred1-TTY</td>
<td>Mumberg, Müller and Funk (1995)</td>
</tr>
<tr>
<td>p414-TEF1p-Cas9-CYC1t</td>
<td>CEN6/ARS4 ampr TRP1 PTEF1-Cas9-TCYC</td>
<td>DiCarlo et al. (2013)</td>
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<td>MB4917</td>
<td>CEN6 ampr URA3</td>
<td>Zelle et al. (2010)</td>
</tr>
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<td>pROS11</td>
<td>2μm amdSYM gRNA-CAN1.Y gRNA-ADE2.Y</td>
<td>Mans et al. (2015)</td>
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<tr>
<td>pUDC156</td>
<td>CEN6 URA3 PTEF1-Cas9-TCYC</td>
<td>This study</td>
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<tr>
<td>pUDR127</td>
<td>2μm amdSYM gRNA-IMAS gRNA-IMA1,2,3,4</td>
<td>de Kok et al. (2011)</td>
</tr>
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<td>pUDI035</td>
<td>Integration plasmid LEU2 Pfre1-MAL11-TCYC</td>
<td>This study</td>
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<td>pUDI084</td>
<td>Integration plasmid LEU2 empty vector</td>
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<td>pUDI044</td>
<td>2μm URA3 Pfre1-MAL2-TADH1</td>
<td>de Kok et al. (2011)</td>
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<td>pUDE260</td>
<td>2μm URA3 empty vector</td>
<td>This study</td>
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<td>pUDE248</td>
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<td>pUDE249</td>
<td>2μm URA3 Pfre1-IMA3,4-TCYC</td>
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<td>pUDE340</td>
<td>2μm URA3 Pfre1-IMA5-TCYC</td>
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Plasmid construction

Plasmid pUDC156 (Table 2) was assembled by in vivo homologous recombination of a plasmid backbone and an insert fragment containing a yeast expression cassette for the cas9 gene from Streptococcus pyogenes (Kuijpers et al. 2013). The plasmid backbone was amplified from MB4917 (Zelle et al. 2010) with primers 7833 and 4697 and the cas9-expression cassette was amplified from p414-TEF1p-Cas9-CYC1t (DiCarlo et al. 2013) with primers 1768 and 7236 (Table S1, Supporting Information). Both fragments contain 60 bp overlaps with each other and were assembled by co-transformation to S. cerevisiae strain IMX469 resulting in IMZ571. Plasmid pUDR127 (Fig. 1A; Table 2) contains two gRNA cassettes: one for deletion of IMAS (gRNA-IMAS) and one targeting sequence shared by IMA1, IMA2, IMA3 and IMA4 (gRNA-IMAs). This plasmid was constructed via Gibson assembly of three fragments: two gRNA cassettes overlapping with each other in the 2 μm replicon and a plasmid backbone. The
plasmid backbone was obtained via PCR amplification, using pROS11 (Mans et al. 2015) as template with primer 6005 (Table S1). The gRNA cassette gRNA-IMA5 was obtained via PCR from pROS11 with primers 8761 and 5975, and cassette gRNA-IMAs was obtained via PCR from pROS11 with primers 8759 and 5974 (Table S1). Plasmid pUDI084 was made by removal of the MAL11 coding sequence from pUDI035 (de Kok et al. 2011) using SpeI and Nhel restriction sites and subsequent recircularisation of the plasmid using T4 DNA ligase (Table 2). Similarly, pUDE260 was constructed by removal of PGmβ from pUDE063 (de Kok et al. 2011) using PvuII sites and subsequent recircularisation of the plasmid (Table 2). The gRNA-reinsertion plasmids (pUDI427 to pUDE430) were made by Gibson assembly of an gRNA expression cassette and a plasmid backbone. The latter was obtained via PCR with p426-GPD (Mumberg, Müller and Funk 1995) as a template, using primer pairs 7823 and 5974 and 5975 and 7812, which amplify the backbone in two parts to minimise chances of plasmid recircularisation (Table S1). Templates for the gRNA expression cassettes were PCR amplified using genomic DNA from strain IMU048 with primers that are specific to each gRNA (primers 8607 and 8611 for gRNA1; 8608 and 8612 for gRNA2; 8609 and 8613 for gRNA3,4; and 8610 and 8614 for gRNA5). From these templates, the coding sequence of each gRNA could be individually amplified using primer pairs 9302 and 9305 (gRNA1), 9303 and 9306 (gRNA2 and gRNA3,4) and 9304 and 9307 (gRNA5). Plasmids were transformed to E. coli for storage and amplification, where necessary after isolation of in vivo assembled plasmids from yeast cultures.

**Strain construction**

Saccharomyces cerevisiae transformations were carried out according to Gietz and Woods (2002) using 1 μg DNA, unless specified otherwise below. Transformants were selected on agar plates containing SM with 20 g L⁻¹ glucose. The following components were added when necessary: G418 200 mg L⁻¹; uracil 0.15 g L⁻¹; leucine 0.5 g L⁻¹. Cells expressing the amdSYM marker were selected on plates according to Solis-Escalante et al. (2013). Strain IMK289 (de Kok et al. 2011) was transformed with a deletion cassette containing loxP-KanMX4-loxP marker. This cassette was amplified from plasmid pUG6 using primers 1482 and 1483 that has homology to the sequence outside the SUC2 open reading frame. The resulting strain was named IMK291. To construct MAL11-expressing strains, vector pUDI035 (Table 2) was linearised with BstEII (restriction site at LEU2 marker) and transformed into IMK291, resulting in strain IMX469 (Table 1). Transformation of pUDE260 and pUDE044 into IMX469 resulted in IMU048 and IMU055, respectively. Linearisation of pUDI084 (Table 2) with BstEII and transformation into IMK291 resulted in IMX470. Transformation of pUDE260 and pUDE044 into IMX470 resulted in IMU051 and IMU054, respectively. IMK700 was made via co-transformation of IMZ571 with 1 μg of plasmid pUDR127 together with 4 μg of each dsDNA repair fragment: one for IMAS and another one for all the other gRNA genes (Fig. 1). Repair fragments were obtained by annealing two complementary PAGE-purified single-stranded oligonucleotides according to Mans et al. (2015): 8592 and 8593 for gRNA1-4 and 8763 and 8764 for IMAS (Table 2). In order to delete gRNA genes from strains evolved on sucrose—IMS422, IMS423 and IMS424—pUDE260 was cured from these strains by cultivation on SM plates with 20 g L⁻¹ glucose and 1 g L⁻¹ 5'-fluoroorotic acid (Boeke, La Courte and Fink 1984), resulting in strains IMS517, IMS518 and IMS519, respectively. Transformation of pUDC156 into these three strains yielded strains IMS604, IMS605 and IMS606, respectively. Finally, transformation of pUDR127 and repair fragments for IMAS and gRNA1-4 (as mentioned above) into these three strains yielded

![Figure 1. Strategy for deletion of the gRNA genes using a shared Cas9 target site. (A) Plasmid pUDR127, that contains two gRNA-cassettes: ‘gRNA-IMAs’ (in red) with a target sequence to cleave all gRNA genes except gRNA5 and ‘gRNA-IMA5’, in purple. The SNR52 promoter and SUP4 terminator are shown in green and yellow, respectively. Sequences encoding structural RNAs are shown in grey. 2 μm: origin of replication in yeast; pMB1: origin of replication in bacteria; ampR: cassette for ampicillin resistance in E. coli; amdSYM: dominant marker that allows use of acetamide as nitrogen source. (B) Illustration of the shared Cas9 target sequences among the gRNA genes. The coding sequence of each gRNA is represented by black lines. The red boxes correspond to the shared target sequence. The dark- and light-grey fragments are the homology regions (60 bp) up- and downstream of the target site where the repair fragment (120 bp) can recombine. In view of its sequence divergence from the other gRNA genes, an exclusive target sequence, in purple, and repair sites, in dark and light green, were chosen for inactivation of gRNA5.](image-url)
strains IMK717, IMK718 and IMK719. For re-insertion of the IMA genes on multicopy plasmids, pUDC156 was cured from IMK700 resulting in IMK743. Then, each IMA overexpression plasmid (named pUDE427-pUDE430, Table 2) was transformed into IMK743 resulting in strains IMZ620-623.

**Medium and cultivation**

Shake-flask cultures were performed in 500 mL shake flasks containing 100 mL of SM with 20 g L$^{-1}$ sucrose, in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ, USA) set at 200 rpm and at 30°C under an air atmosphere. For growth rate determinations, cells were inoculated in SM with 20 g L$^{-1}$ glucose from a frozen stock culture. After reaching stationary phase, the culture was transferred to SM with 20 g L$^{-1}$ sucrose (initial OD$_{660nm}$ = 0.2) and incubated until exponential growth was observed. Exponentially growing cultures were then transferred to fresh medium (initial OD$_{660nm}$ = 0.2) and samples were taken hourly until stationary phase was reached. Optical density at 660 nm was measured with a Libra S11 spectrophotometer (Biochrom, Cambridge, UK). Specific growth rates were calculated from at least five data points.

**Laboratory evolution**

Sequential batch cultivation of strain IMU048 was performed in 10 mL SM (Verduyn et al. 1992) with 20 g L$^{-1}$ sucrose, in 50 mL polypropylene tubes (Greiner Bio-One, Frickenhausen, BW) in an Innova incubator shaker (New Brunswick Scientific), at 200 rpm and at 30°C. At the end of each cultivation cycle (OD$_{660nm}$, 3–4.2), 0.1 mL of culture was transferred to fresh medium to start a next cycle. In total, 30 transfers were carried out in 70 days, which accounts for ~ 180 generations. One single-colony isolate from each evolution line was obtained by restreaking three times on non-selective medium (YPD) plates.

**Sucrose and glucose determination**

Concentrations of sucrose and glucose in culture supernatants were analysed by high-performance liquid chromatography (Agilent 1100 HPLC, Agilent Technologies, Santa Clara, CA), using an Aminex HPX-87H ion exchange column (BioRad, Richmond, CA) coupled to a refractive-index detector and eluted with 0.5 mM H$_2$SO$_4$ at 0.8 mL min$^{-1}$ and at 40°C. This temperature was used instead of the regularly applied temperature of 60°C to avoid sucrose hydrolysis during analysis.

**Enzyme activity assays**

For enzyme activity assays, shake-flask cultures on SM with 20g L$^{-1}$ sucrose were harvested during exponential growth (OD$_{660nm}$ = 3–4). For strains IMU054, IMU055, IMZ620, IMZ621, IMZ622 and IMZ623, ethanol (20 g L$^{-1}$) was used as carbon source instead of sucrose. For strains IMK700, IMK716, IMK717 and IMK718, 20 g L$^{-1}$ ethanol was used as carbon source plus 20 g L$^{-1}$ sucrose as inducer. Samples were harvested, washed and prepared for sonication according to Postma et al. (1989). Cell extracts were prepared by sonication with 0.7 mm glass beads at 0°C for 2 min at 0.5 min intervals with an MSE sonicator (150 W output; 8 µm peak-to-peak amplitude). Unbroken cells and debris were removed by centrifugation (4°C, 20 min, 47 000 × g). The supernatant was used for enzyme activity assays. For measurement of extracellular enzyme activity, 100 mL of exponentially growing cells (OD$_{660nm}$ = 3–4) was centrifuged (4°C, 10 min, 5000 × g), the supernatant was concentrated up to 200 times with a Vivaspin® 20 filter with a 10 000 MW cut-off (Sartorius Stedim, Aubagne, France) and dialysed overnight against 10 mM potassium-phosphate buffer (pH 7.5) at 4°C. Protein levels in extracellular samples and cell extracts were determined with the Lowry assay (Lowry et al. 1951). Sucrose or isomaltose hydrolytic activity was measured at 30°C by monitoring the reduction of NADP$^+$ at 340 nm in a 1 mL reaction mixture containing 50 mM imidazole-HCl (pH 7.0), 1 mM NADP$^+$, 12.5 mM MgCl$_2$, 1 mM ATP, 3.5 units hexokinase, 3.5 units glucose-6-phosphate dehydrogenase and 10–40 µL cell extract. The reaction was started by the addition of 100 mM of substrate. An extinction coefficient of 6.3 mM$^{-1}$ was used for NADPH.

**Real-time quantitative PCR**

Exponentially growing cultures (OD$_{660nm}$ = 3–4) in 100 mL SM (Verduyn et al. 1992) with 20 g L$^{-1}$ of sucrose were harvested as described previously (Piper et al. 2002). RNA was extracted using the hot-phenol method (Schmitt, Brown and Trumpower 1990), and RNA quality was assayed by electrophoresis using an Agilent BioAnalyzer 2100 (Agilent Technologies). Genomic DNA elimination was performed from 2 µg of total RNA in a 28 µL reaction using the QuantiTec Reverse Transcription kit (Qiagen, Düsseldorf, Germany). Reagents for cDNA synthesis from the same kit were added up to a final volume of 40 µL following the manufacturer’s instructions. A portion of 6 µL of cDNA solution diluted 150 times was used in 20 µL qPCR mix that additionally included 10 µL of Rotor-Gene SYBR Green PCR Master Mix (Qiagen) and forward and reverse primers (1 µM each) (Table S1). A Rotor-Gene Q (Qiagen) was used with the following sequence: denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 10 s and extension at 72°C for 20 s. A melting curve up from 60°C to 95°C was performed to verify primer specificity. PCR efficiency of each primer was determined by a dilution series using a pool of cDNA from different samples. Transcript levels of the ‘housekeeping’ gene UBC5 (Teste et al. 2009) were used for data normalisation. Threshold cycles (CT) were exported from Rotor-Gene Q software (version 2.0.2) and analysed using the REST-2009 algorithm (Pfaffl, Horgan and Dempfle 2002). One of the two biological replicates of CEN.PK113-7D was chosen as reference condition (expression ratio = 1).

**Sanger and whole-genome sequencing**

Sanger sequencing of PCR products of each IMA gene was performed at BaseClear BV (Leiden, The Netherlands). The PCR products were obtained with genomic DNA extracted from strains IMS422, IMS423 and IMS424 and following primers: 8607 and 8611 (IMA1), 8608 and 8612 for (IMA2), 8609 and 8613 for (IMA3,4) and 8610 and 8614 (IMA5) (Table S1). IMA3 and IMA4 were treated as a single gene (IMA3,4) since their coding sequences are identical (Teste, François and Parrou 2010).

DNA for whole genome sequencing was extracted using Qiagen 100G kit following the manufacturer’s protocol (Qiagen, Hilden, Germany). Whole-genome sequencing was performed by Novogene (Beijing, China). A library of 350-bp genomic fragments was created and sequenced paired end (150-bp reads). A minimum data quantity of 4000 MB was generated per strain, representing a minimum 330-fold coverage. The data analysis was performed as described by van den Broek et al. (2015). The sequencing data of the parental strain, IMU048, and of the three
evolved isolates, IMS422, IMS423 and IMS424, were deposited at NCBI under the BioProject ID: PRJNA353914.

RESULTS

Experimental design to eliminate non-biological sucrose hydrolysis

Although sucrose is generally stable when dissolved in water, under certain conditions it can be hydrolysed into fructose and glucose, such as for instance by acid catalysis in low pH solutions (Kriebel 1935; Wolfenden and Yuan 2008). To investigate possible artefacts created by such non-biological hydrolysis of sucrose, strain IMU051 (malΔ mphΔ suc2Δ), which cannot grow on sucrose, but can grow on hexoses, was incubated in SM shake flasks at pH values between 2 and 7 (Fig. 2). At pH values of 5–7, no growth was observed (Fig. 2A). At pH 3, linear growth was observed (Fig. 2A), which was consistent with the occurrence of sucrose hydrolysis in a sterile culture at this acidic pH (Fig. 2B). While sucrose hydrolysis also occurred at pH 2 (Fig. S2, Supporting Information), no growth was observed at this pH value, since Saccharomyces cerevisiae CEN.PK113-7D cannot grow at this low pH (Della-Bianca et al. 2014). To avoid a contribution of non-biological hydrolysis of sucrose to the initiation of growth, all further growth experiments in this study were done in cultures with an initial pH of 6. For those cultures that did show growth, the pH eventually decreases to values below 3. However, since this drop only occurs towards the end of the exponential growth, this did not influence the initiation of growth.

Residual sucrose hydrolysis encoded by unknown genes

To investigate what is needed to completely abolish both sucrose transport and hydrolysis in S. cerevisiae CEN.PK102-3A, a strain was constructed in which the following genes were deleted: SUC2 (encoding invertase); MAL12, MAL22 and MAL32 (encoding maltases); and MAL11, MAL21, MAL31, MPH2, MPH3 (encoding transporters). The resulting strain (IMU051; malΔ mphΔ suc2Δ) did not show any growth over a period of up to 2 months in SM with sucrose as the sole carbon source (Table 3). However, for future metabolic engineering strategies concerning sucrose
metabolism, it is important to understand whether this inability to grow is caused by lack of transport or lack of sucrose hydrolysis. To investigate this, three new strains were constructed: one expressing the transporter Mal11 (IMU048; malΔ mphΔ suc2Δ MAL11), one expressing the α-glucosidase Mal12 (IMU054; malΔ mhpΔ suc2Δ MAL12) and a control strain expressing both genes (IMU055; malΔ mhpΔ suc2Δ MAL11 MAL12). The maltase Mal12 was chosen for intracellular cleavage of sucrose rather than the invertase Suc2, since previous work has shown that extracellular Suc2 activity can be detected even if Suc2 is expressed without the secretion signal sequence (Basso et al. 2011).

Combined expression of MAL11 and MAL12 resulted in a maximum specific growth rate of 0.19 h⁻¹ for the control strain IMU055 (Table 3). Even after prolonged incubation of up to 2 months, expression of only the hydrolysing enzyme Mal12 (IMU054) did not enable growth in SM with sucrose as the sole carbon source, indicating that there is no residual sucrose transport activity sufficient to allow growth and that this phenotype is stable. In contrast, the strain that only expressed the transporter Mal11 (IMU048; malΔ mhpΔ suc2Δ MAL11) grew at a maximum specific growth rate of 0.08 h⁻¹ in SM with sucrose, after a lag phase of about 4 days (Table 3). All shake-flask cultivations were performed under an air atmosphere. In line with this observation, an activity of sucrose hydrolysis of 0.48 ± 0.03 μmol mg protein⁻¹ min⁻¹ was detected in cell extracts of this strain. Although lower than the activity of 1.67 ± 0.08 μmol mg protein⁻¹ min⁻¹ observed in the reference strain IMU055, this activity was sufficient to sustain growth on sucrose (Table 3).

Laboratory evolution enables sucrose hydrolysis and increased IMA expression

To investigate which genes encode the enzymes responsible for residual sucrose hydrolysis activity in strain IMU048 (malΔ mhpΔ suc2Δ MAL11), this strain, which grows slowly in a sucrose-based medium (μ = 0.08 h⁻¹, Table 3), was subjected to sequential batch cultivation in SM with sucrose 2% (w/w) as sole carbon source. After 30 transfers (70 days, corresponding to ~180 generations), single-colony isolates (IMU422, IMU423 and IMU424) were obtained from three independent evolution experiments. In addition to a 3-fold increase in maximum specific growth rate (from 0.08 to 0.25–0.26 h⁻¹, Table 3), the lag phase of these strains was shortened from 4 days to 1 day.

Enzyme-activity assays were conducted to investigate whether the increased growth rate of the evolved strains on sucrose correlated with sucrose hydrolytic activity. Indeed, intracellular sucrose hydrolysis activities in the evolved strains were 2- to 3-fold higher (1.10–1.57 μmol mg protein⁻¹ min⁻¹) than in the non-evolved parental strain IMU048 (0.48 μmol mg protein⁻¹ min⁻¹). No extracellular sucrose-hydrolysing activity was detected in cultures of these strains. In contrast, in cultures of the reference strain CEN.PK113-7D, extracellular sucrose activity was 5.40 μmol mg protein⁻¹ min⁻¹ while intracellular activity was only 0.43 μmol mg protein⁻¹ min⁻¹ (Table 3). Activity of isomaltases, which are known to also hydrolyse sucrose (Deng et al. 2014), was measured to investigate a possible contribution of these enzymes to the observed evolved phenotype. Indeed, isomaltase activity was about 1.5–2 times higher in the evolved strains (1.59–2.31 μmol mg protein⁻¹ min⁻¹) compared to IMU048 (1.05 μmol mg protein⁻¹ min⁻¹) (Table 3).

In CEN.PK113-7D, isomaltase activity was only 0.13 μmol mg protein⁻¹ min⁻¹ (Table 3). Since isomaltases cannot hydrolyse maltose (Deng et al. 2014), it was checked if the evolved strains could consume this sugar. After 2 months of incubation in SM with maltose, no growth was observed.

Quantitative real-time PCR was performed to investigate if the differences in sucrose and isomaltose hydrolysis observed among reference, unevolved and sucrose-evolved strains could be explained by differences in gene expression. Expression of IMA2, IMA3 and IMA4 was analysed with a single primer pair since the sequences of these genes are highly similar. No difference in expression ratio was observed for the pool consisting of IMA2, IMA3 and IMA4 transcripts among the strains tested (Fig. 3C). In contrast, IMA1 and IMA5 expression was significantly higher in IMU048 (malΔ mhpΔ suc2Δ MAL11) than in CEN.PK113-7D. Expression of these genes in the evolved strains was even 3- to 5-fold higher than in the unevolved strain IMU48 (Fig. 3A and B). In all analysed strains, the relative expression level of IMA5 was approximately 5–10 times higher than that of IMA1 (Fig. 3A and B).

Deletion of IMA genes is required and sufficient to completely eliminate sucrose hydrolysis

Based on the results shown above, we tested whether deletion of the IMA genes would be sufficient to abolish sucrose consumption in the evolved strains (IMS422, IMS423 and IMS424) and in an unevolved strain (IMZ571; malΔ mhpΔ suc2Δ MAL11-LEU2 can5-URA3) (Table 1). All six copies of IMA genes present in strains IMZ571 and IMS422–424, all derived from strain CEN.PK102-3A (Table 1) (de Kok et al. 2011; Nijkamp et al. 2012), were deleted in a single transformation step using only two gRNAs cloned in a single plasmid. One of these gRNAs targeted IMA5 while the other simultaneously targeted all other IMA genes. None of the resulting strains (named IMK715, IMK717, IMK718 and IMK700, respectively) grew in SM with sucrose as sole carbon source after 10 days of incubation (Table 4) or after a further 2 months of incubation. Both the sucrose and isomaltose hydrolysis activities in the resulting strains were below the detection limit (Table 4).

IMA1 and MAL23-C copy number increased in evolved strains

Sanger sequencing of the IMA genes including their promoter regions did not reveal any mutations (IMA genes from the evolved strains in comparison to that of the parental). Whole genome sequencing of the parental strain IMU048 revealed that, upon sequential cycle of deletions using loxP sites, the strain became aneuploid for CHRII and CHRVII. Regarding CHRII, the parental strain gained an extra copy after deletions using loxP sites, truncating the majority of the left arm. Sequencing of three independently evolved isolates indicated a further increase in copy number of the right arm of CHRII (Fig. 4). Relative to the left arm of the same chromosome (until approximately position 90 000), the region between positions 90 000 and 150 000, which includes the centromere, showed an approximately 2-fold increase in read depth. The right-arm distal end (beyond position 150 000) even showed a 3-fold higher increase in read depth in all three evolved strains relative to the distal end of the left arm (Fig. 4).

Retrotransposons (e.g. YCLIWA15 and YCRC6) located near positions 90 000 and 150 000 may have contributed to translocation/duplication events at these loci. The duplicated CHRII region in the parental strain IMU048 and triplicated in the evolved isolates harboured IMA1 and MAL23-C. Additionally, a region from CHRVII located between retrotransposons (from position
70 700 to 82 000) was found duplicated already in the parental strain and, in the evolved strains, this duplication was lost.

To investigate the impact of an increased expression of IMA genes during growth on sucrose, they were individually overexpressed in a strain in which all known sucrose-hydrolysing activities were inactivated (IMK743; malΔ mphΔ suc2Δ MAL11 imaΔ) (Table 1). All four constructed strains grew in a sucrose-based medium: strains IMZ620 and IMZ621 (overexpression of IMA1 and IMA2, respectively) both grew at a specific growth rate of 0.19 h⁻¹. Accordingly, sucrose and isomaltose hydrolysis activities in these strains were ca. 2 μmol mg protein⁻¹ min⁻¹ (Table 4). Strain IMZ622, which overexpressed IMA3/4, grew at 0.17 h⁻¹, while IMZ623 (IMA5 overexpressed) grew at 0.04 h⁻¹. Sucrose hydrolysis activities in cell extracts of strains IMZ622 and IMZ623 were 0.92 and 0.17 μmol mg protein⁻¹ min⁻¹, respectively (Table 4). No maltose hydrolysis was detected in any of the IMA-overexpressing strains.

**DISCUSSION**

This study confirms the power of laboratory evolution in identifying suppressor mutations of seemingly essential genes (Liu et al. 2015). Prolonged incubation of the malΔ mphΔ suc2Δ MAL11 imaΔ strain for up to 2 months demonstrated that deletion of the IMA genes is both required and sufficient to eliminate growth on sucrose in a strain lacking invertase and maltase activities. Deletion of the disaccharide transporter genes MAL11, MAL21, MAL31, MPH2 and MPH3 similarly resulted in a sucrose-negative phenotype that was stable for over 2 months of incubation in a sucrose-containing medium. The phenotypic stability of the sucrose-negative strains generated in this study makes them a suitable platform for use in diverse applications: (i) screening heterologous disaccharide transporters (e.g. characterisation of plant sucrose transporters in yeast; study maltose/maltotriose transporters relevant for beer brewing (Alves et al. 2008)); (ii) screening and characterisation of disaccharide hydrolases and phosphorylases; (iii) metabolic engineering of disaccharide metabolism for improving the production of biofuels and other bio-based chemicals.

Before laboratory evolution for improved growth on sucrose, strain IMU048 (malΔ mphΔ suc2Δ MAL11) already showed a much higher expression of IMA1 and IMA5 compared to CEN.PK113-7D. A main difference between IMU048 and CEN.PK113-7D is the location of sucrose hydrolysis. In the reference strain CEN.PK113-7D, sucrose is predominantly hydrolysed extracellularly (Basso et al. 2011), whereas in IMU048 sucrose is imported via Mal11 (proton symporter) and hydrolysed in the cytosol. The presence of intracellular sucrose could possibly activate transcription factors such as the MAL activators MALx3 (Alamæ et al. 2003; Weinhandl et al. 2014). It is important to clarify that the deletions carried out in this study included maltases and MAL transporters but not the MAL activators (MAL13, MAL23-C and MAL33). MAL23-C (known as MAL2-8f) is constitutively expressed (Gibson et al. 1997) but might undergo posttranslational inhibition by chaperones, as has been shown for Mal63, for which the inhibition is relieved when maltose is present (Bali et al. 2003; Ran, Bali and Michels 2008). Teste, François and Parrou (2010) showed that the IMA1 and IMA5 promoter regions contain one and three binding sites for MALx3, respectively. The other IMA promoters do not contain binding sites for MALx3 (Teste, François and Parrou 2010). The same authors showed that maltose induces higher expression levels of IMA5 than of IMA1, and has no effect on the expression levels of IMA2, IMA3 and
Table 4. Maximum specific growth rates of \textit{S. cerevisiae} strains grown in shake flasks containing SM (initial pH 6.0) with 20 g L\(^{-1}\) sucrose as sole carbon source. For enzymatic activity determination, strains were grown in SM with 20 g L\(^{-1}\) ethanol as sole carbon source (sucrose was added besides ethanol to the culture of strains IMK716-18 as an inducer). Averages and mean deviations were obtained from duplicate experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Growth on sucrose (h(^{-1}))</th>
<th>Intracellular sucrose hydrolysis (µmol mg protein(^{-1}) min(^{-1}))</th>
<th>Intracellular isomaltose hydrolysis (µmol mg protein(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMK716</td>
<td>IMU048 evolved #1 cas9 imaΔ</td>
<td>No growth(^a)</td>
<td>B.D.</td>
<td>B.D.</td>
</tr>
<tr>
<td>IMK717</td>
<td>IMU048 evolved #2 cas9 imaΔ</td>
<td>No growth(^a)</td>
<td>B.D.</td>
<td>B.D.</td>
</tr>
<tr>
<td>IMK718</td>
<td>IMU048 evolved #3 cas9 imaΔ</td>
<td>No growth(^a)</td>
<td>B.D.</td>
<td>B.D.</td>
</tr>
<tr>
<td>IMZ620</td>
<td>malΔ mphΔ suc2Δ MAL11 imaΔ IMA1</td>
<td>0.19 ± 0.01</td>
<td>2.38 ± 0.03</td>
<td>4.60 ± 0.54</td>
</tr>
<tr>
<td>IMZ621</td>
<td>malΔ mphΔ suc2Δ MAL11 imaΔ IMA2</td>
<td>0.19 ± 0.01</td>
<td>2.88 ± 0.28</td>
<td>1.95 ± 0.03</td>
</tr>
<tr>
<td>IMZ622</td>
<td>malΔ mphΔ suc2Δ MAL11 imaΔ IMA3,4</td>
<td>0.17 ± 0.01</td>
<td>0.92 ± 0.03</td>
<td>0.64 ± 0.14</td>
</tr>
<tr>
<td>IMZ623</td>
<td>malΔ mphΔ suc2Δ MAL11 imaΔ IMA5</td>
<td>0.04 ± 0.01</td>
<td>0.17 ± 0.03</td>
<td>6.11 ± 0.18</td>
</tr>
</tbody>
</table>

\(^a\) Incubation period: 10 days.
B.D.: Below detection limit, i.e. <0.03 µmol mg protein\(^{-1}\) min\(^{-1}\).

IMA4. The strongly increased transcription levels of \textit{IMA1} (110-fold) and \textit{IMA5} (950-fold) in IMU048, relative to those in CEN.PK113-7D (Fig. 3A and B), but not of \textit{IMA2,3,4} (Fig. 3C), is in line with the hypothesis that sucrose activates the MAL-activators. Extra-cellular glucose and fructose released in cultures of the strain CEN.PK113-7D by the action of Suc2 might further contribute to the repression of the MAL activators (Hu et al. 1995; Horák 2013) in this reference strain.

As mentioned above, the complement of genes belonging to the disaccharide hydrolase gene families varies greatly among \textit{Saccharomyces cerevisiae} strains. With even closely related strains showing a high degree of genetic variation, it is important to have access to the genome sequence of the immediate parental strain that is used as a platform on which a metabolic engineering strategy will be implemented. The importance of strain-dependent gene contents is exemplified by a literature debate on the number of binding sites for MALx3 in the promoters of \textit{IMA1} and \textit{IMA5}. In contrast to Teste, François and Parrou (2010), Pougach et al. (2014) stated that the IMA5 promoter only has a single binding site for MALx3. In our study, Sanger sequencing of the promoter region of IMA5 confirmed the presence of three MALx3 binding sites previously identified by Teste, François and Parrou (2010); the motif CGGN(9)CGG was found at positions –141 and –543 and motif MGCN(9)MGS at position –516 (Sirenko, Ni and Needleman 1999). This confirmation does not exclude the possibility that the data of Pougach et al. (2014) are correct, since they used a different \textit{S. cerevisiae} strain (KV5000, which originates from BY4741, an S288c-derived strain) whilst Teste, François and Parrou (2010) used the CEN.PK113-7D strain.

After laboratory evolution, expression of both \textit{IMA1} and \textit{IMA5} increased by 3-fold compared to the unevolved IMU048. Whole-genome sequencing showed that the strain subjected to evolution (IMU048) already contains a duplication of a large part of CHRIII, which starts before the centromere and extends until the right end of the chromosome (Fig. 4). Likewise, CHRVII of IMU048 contains a duplicated region between retrotransposon sites that was lost after the evolution. Therefore, genes located in this CHRVII region might not contribute to sucrose consumption. Although such aneuploidy often go undetected, this mechanism has also been previously identified in a fraction of deletion mutants generated by the \textit{Saccharomyces} genome deletion consortium (Winzeler et al. 1999; Hughes et al. 2000). Several cases reported that the extra chromosome harboured a close homologue of the gene deleted. In our study, deletion of MAL31 and MAL32 may have led to the aneuploidy of CHRIII that harbours the MAL2 locus. Additionally both unevolved (IMU048) and evolved strains (IMS422-4) carried structural variant of the CHRIII. Such rearrangement, especially at chromosome 3, has been extensively reported in the literature as result of recombination between retrotransposons located around positions 90 000 and 150 000 (Mieczkowski, Lemoine and Petes 2006; de Kok et al. 2012). In all the three evolved strains obtained in this study, a third copy of the mentioned region was found (Fig. 4). This region not only contains IMA1 but also MAL23-C. If, as discussed above, sucrose indeed activates MAL23-C, IMA1 expression level could be increased by increase in copy number of the gene encoding its activator, MAL23-C. While in this study overexpression of IMA1 was shown to result in faster growth on sucrose, IMA5 was shown to be predominantly an isomaltose and overexpression resulted in very slow growth and low intracellular sucrose hydrolysis activity (Table 4) (Deng et al. 2014). It therefore seems likely that...
the increased expression of IMAS is collateral to the upregulation of MAL23p.

In addition to the scientific value of the sucrose-negative strain platform, this study demonstrates how a single CRISPR targeting sequence can be used to simultaneously delete multiple genes in _S. cerevisiae_. This strategy allows the deletion and/or modification of entire gene families in a single transformation event.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSYR online.

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**Conflict of interest.** None declared.

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