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A CRISPR/Cas9-based exploration into the elusive mechanism for lactate export in *Saccharomyces cerevisiae*

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Abstract (199/200 words)

CRISPR/Cas9-based genome editing allows rapid, simultaneous modification of multiple genetic loci in *Saccharomyces cerevisiae*. Here, this technique was used in a functional analysis study aimed at identifying the hitherto unknown mechanism of lactate export in this yeast. First, an *S. cerevisiae* strain was constructed with deletions in 25 genes encoding transport proteins, including the complete aqua(glycero)porin family and all known carboxylic-acid transporters. The 25-deletion strain was then transformed with an expression cassette for *Lactobacillus casei* lactate dehydrogenase (*LcLDH*). In anaerobic, glucose-grown batch cultures, this strain exhibited a lower specific growth rate (0.15 vs. 0.25 h^{-1}) and biomass-specific lactate production rate (0.7 vs. 2.4 mmol (g biomass)$^{-1}$ h^{-1}) than an *LcLDH*-expressing reference strain. However, a comparison of the two strains in anaerobic glucose-limited chemostat cultures (dilution rate 0.10 h^{-1}) showed identical lactate production rates. These results indicate that, although deletion of the 25 transporter genes affected the maximum specific growth rate, it did not impact lactate export rates when analysed at a fixed specific growth rate. The 25-deletion strain provides a first step towards a 'minimal transportome' yeast platform, which can be applied for functional analysis of specific (heterologous) transport proteins as well as for evaluation of metabolic engineering strategies.

Introduction

Based on current estimates, membrane transport in *Saccharomyces cerevisiae* involves approximately 300 proteins, of which 139 occur in the plasma membrane (Brohée *et al.* 2010). In cases where membrane translocation of a compound is mediated by one or a few transporter protein(s), accurate determination of mutants in which the corresponding structural gene(s) have been inactivated can aid the identification of transporter function and kinetics (Roman 1956; Grenson *et al.* 1966; Fisher 1969; Silver and Eaton 1969; Whelan, Gocke and Manney 1979). When

transport of a single metabolite involves multiple transporters whose functions are not fully redundant, disruption of a single gene can cause a reduced fitness. In such cases, laboratory evolution can (partially) recover the reduced fitness. Subsequent analysis of the responsible suppressor mutations can then help to identify alternative transporters (De Kok *et al.* 2012). When the presence or absence of a transporter for a specific molecule does not result in a phenotype that can be easily analysed (e.g. growth/no-growth), synthetic selection systems such as ligand-responsive biosensors be engineered for screening of functional transporters (Genee *et al.* 2016). Alternatively, identification of genes whose expression is upregulated in the presence of the molecule of interest, for example via transcriptomics, could provide insight in the genes responsible for uptake or export of this compound (for a recent example see Thompson *et al.* 2016).

When an organism harbours a large number of transporters that are able to transport a single compound, single-gene deletion experiments are of very limited value for functional analysis (Delneri *et al.* 2000). The paradigm for addressing such a challenge in yeast research is the study by Wieczorke et al (1999) on the hexose-transporter (*HXT*) gene family in *S. cerevisiae*. These authors showed that sequential deletion of the hexose-transporter genes *HXT1*, *HXT2*, *HXT4*, *HXT5* and *HXT8-16/17* did not cause a distinct growth defect on glucose, fructose or mannose. Instead, growth on glucose was only completely abolished after subsequent deletion of the remaining *HXT3*, *HXT6* and *HXT7* genes (Wieczorke *et al.* 1999). The resulting 'HXT null' strain platform has become an invaluable platform for functional analysis of native and heterologous hexose and pentose transporter genes (for examples see Buziol *et al.* 2002, Wieczorke *et al.* 2003 and Subtil and Boles 2011, for a complete overview see Solis-Escalante *et al.* 2014). At the time of its construction, deletion of so many genes represented a herculean task. Later analysis showed that using multiple rounds of Cre-*LoxP*-based marker-gene recovery had, in fact, created several chromosomal translocations (Solis-Escalante *et al.* 2014).

In contrast to the advanced state of research on *HXT* genes in *S. cerevisiae*, research on the transport of other simple metabolites in this yeast is still in its infancy (Kell *et al.* 2015) and the mechanism by which lactate is exported from yeast cells is a particularly elusive metabolic conundrum. In *S. cerevisiae*, import of lactate can be catalysed by the major facilitator superfamily (MFS) lactate transporter Jen1 and the acetate transporter Ady2 (Cássio, Leao and Van Uden 1987; Casal *et al.* 1999; Paiva *et al.* 2004; Ramos, Sychrová and Kschischo 2016) and combined deletion of *JEN1* and *ADY2* eliminates growth on lactate as the sole carbon source at pH 5.0 (De Kok *et al.* 2012). While a role of Jen1 in lactate export has been proposed (Porro *et al.* 1999; Branduardi *et al.* 2006), the combined deletion of *JEN1* and *ADY2* does not affect lactate-production rates by *S. cerevisiae* strains expressing a heterologous NAD$^+$-linked lactate dehydrogenase (*LDH*) (Pacheco *et al.* 2012).

Understanding the mechanism of lactate export in *S. cerevisiae* is of fundamental interest and, moreover, highly relevant for industrial production of lactic acid with engineered yeast strains (Dequin and Barre 1994; Porro *et al.* 1995; Van Maris *et al.* 2004a). Engineered 'homolactic' strains of *S. cerevisiae*, in which the three pyruvate decarboxylase genes have been replaced by a heterologous *LDH* gene, strictly require aeration for growth (Van Maris *et al.* 2004b; Abbott *et al.* 2009). This oxygen requirement indicates that conversion of glucose to (extracellular) lactate does not result in a net formation of ATP and has been interpreted as reflecting a net ATP requirement for lactate export (**Figure 1**) (Van Maris *et al.* 2004b; Abbott *et al.* 2009). Already before the gene deletion study by Pacheco *et al.* (2012), Jen1 and Ady2 were unlikely candidates for a role as lactate exporter. Both transporters are reported to mediate electroneutral symport of a carboxylate anion with a proton (Cássio, Leao and Van Uden 1987; Casal and Cardoso 1996; Casal *et al.* 1999), which is not compatible with a net stoichiometric requirement for ATP (Van Maris *et al.* 2004a; Abbott *et al.* 2009).

At least three distinct mechanisms can be envisaged for energy-dependent transport of lactate (Van Maris *et al.* 2004a); i) transport via an ABC transporter that couples hydrolysis of ATP to export of the undissociated lactic acid or the lactate anion or ii) (facilitated) diffusion of the lactate anion accompanied by export of the associated proton via the plasma-membrane proton ATPase (which, in *S. cerevisiae*, has a stoichiometry of 1 H^+ per ATP; Van Leeuwen *et al.* 1992 and Weusthuis *et al.* 1993), or iii) antiport of lactic acid or lactate with a cation (most likely H^+ or Na^+) and subsequent cation-extrusion via a P-type ATPase to maintain proton-motive force and intracellular pH homeostasis.

The ABC transporter Pdr12 is involved in export of lipophilic carboxylic acids (Piper *et al.* 1998; Holyoak *et al.* 1999; Bauer *et al.* 2003; Hazelwood *et al.* 2006; Ullah *et al.* 2013; Nygård *et al.* 2014). However, a role of Pdr12 or other ABC transporters in export of lactate has not been demonstrated and overexpression of *PDR12* made cells more sensitive to lactic acid(Nygård *et al.* 2014). Similarly, although the aquaglyceroporin Fps1 mediates diffusion of other carboxylic acids over the yeast plasma membrane (Mollapour and Piper 2007), a similar transport mechanism for the lactate anion has not been described. Additionally, the strongly reduced uptake of acetic acid via Fps1 at high pH values suggests that the undissociated carboxylic acid rather than the anion is the substrate(Mollapour and Piper 2007), which would not be compatible with a net ATP requirement for lactate export via this aquaglyceroporin.

Polar carboxylic acid anions such as lactate are generally assumed not to freely diffuse over membrane lipid bilayers at physiologically relevant rates (Cássio, Leao and Van Uden 1987; Lambert and Stratford 1999). Based on this assumption, at least one lactate transporter, responsible for energy-coupled lactate export, remains to be identified (Van Maris *et al.* 2004b; Abbott *et al.* 2009). Hirasawa *et al.* (2013) addressed this challenge by transforming 4802 single-gene deletion mutants of *S. cerevisiae* with an expression cassette for an *LDH* gene. Subsequently,

these authors quantified the effect of the deletion on the lactate production rates. Only one transporter gene deletion, in *PDR12*, caused a > 90 % reduction of final lactate titers. However, this effect was probably an artefact caused by the use of an auxotrophic strain background (Bauer *et al.* 2003) and the other single-gene deletions of transporter encoding genes did not have a similarly strong impact on lactate export. Apparently, the lactate exporter gene was not amongst the 4802 genes included in the screening or, alternatively, multiple, functionally redundant transporters mediate lactate export.

The recent development and implementation of CRISPR/Cas9-based genome editing has facilitates the simultaneous introduction of genetic modifications in multiple yeast genes (at least up to six; Bao *et al.* 2015; Horwitz *et al.* 2015; Mans *et al.* 2015). The aim of the present study was to investigate the elusive molecular basis for lactate export in *S. cerevisiae*. Candidate lactate transporters were selected based on their ability to transport other carboxylic acids, involvement in carboxylic-acid tolerance and similarity to known carboxylic acid transporter proteins. Additional target genes were selected because their deletion was reported to affect lactate production in a strain expressing *LDH* (Hirasawa *et al.* 2013). The selected transporters were then all deleted in a single strain by CRISPR-Cas9-mediated genome editing. Subsequently, an *LDH* gene was introduced and the biomass-specific lactate production rate of the resulting strain was compared to that of an isogenic control strain containing the same *LDH* expression cassette but no transporter deletions.

Materials and methods

Strains, growth conditions and storage

The *S. cerevisiae* strains used in this study (**Table 1**) share the CEN.PK genetic background (Entian and Kötter 2007; Nijkamp *et al.* 2012) and are available upon request. Shake flask cultures were grown at 30°C in 500 mL flasks containing 100 mL synthetic medium (SM) (Verduyn *et al.* 1992)

with 20 g L⁻¹ glucose in an Innova incubator shaker (Eppendorf, Hamburg, Germany) set at 200 rpm. After introduction of the lactate dehydrogenase gene, a mixture of 2% (v/v) glycerol and 2% (v/v) ethanol was used as a carbon source during strain construction (see below). When required, auxotrophic requirements were complemented via addition of 150 mg L⁻¹ uracil, 100 mg L⁻¹ histidine, 500 mg L⁻¹ leucine, 75 mg L⁻¹ tryptophan (Pronk 2002) or by growth in YP medium (demineralized water, 10 g L⁻¹ Bacto yeast extract, 20 g L⁻¹ Bacto peptone). For plate cultivation 2% (w/v) agar was added to the medium prior to heat sterilization. Frozen stocks were prepared by addition of sterile glycerol (30% v/v) to exponentially growing shake-flask cultures of *S. cerevisiae* or overnight cultures of *E. coli* and 1 mL aliquots were stored aseptically at -80 °C .

General molecular biology techniques

PCR amplification with Phusion Hot Start II High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA) was performed according to the manufacturer's instructions using PAGE-purified oligonucleotide primers (Sigma-Aldrich, St. Louis, MO). Diagnostic PCR was done via colony PCR on randomly picked yeast colonies, using DreamTaq (Thermo Fisher Scientific) and desalted primers (Sigma-Aldrich). The primers used to confirm successful deletions can be found in **Table S1**. Yeast genomic DNA was isolated according to Lõoke *et al* (Lõoke, Kristjuhan and Kristjuhan 2011). DNA fragments obtained by PCR were separated by gel electrophoresis on 1% (w/v) agarose gels (Thermo Fisher Scientific) in TAE buffer (Thermo Fisher Scientific) at 100 V for 30 min. Fragments were excised from gel and purified by gel purification (Zymoclean D2004, Zymo Research, Irvine, CA). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich) according to the supplier's manual. Restriction analysis was performed using FastDigest enzymes (Thermo Scientific) according to the manufacturer's manual. Plasmid assembly was performed using the Gibson Assembly Cloning kit (New England Biolabs, Ipswich, MA) according to the suppliers protocol, but downscaled to 5 µL total volume. *E. coli* DH5α (18258-012, Thermo Fisher Scientific) or XL1-Blue (GE Healthcare Life Sciences, Uppsala, Sweden) was used for chemical transformation

(T3001, Zymo Research) or for electroporation. Chemical transformation was done according to the supplier's instructions. Electroporation was done in a 2 mm cuvette (165-2086, BioRad, Hercules, CA) using a Gene PulserXcell Electroporation System (BioRad), following the manufacturer's protocol. Electrocompetent DH5α cells were prepared according to BioRad's protocol, except for the use of LB medium without NaCl when pre-growing the cells.

Plasmid construction

For CRISPR/Cas9-based deletion of the transporter genes, plasmids containing two guide RNA (gRNA) sequences were constructed as described previously (Mans *et al.* 2015). **Table S1** lists the primers used for the amplification of the 2µm fragment for each of the double gRNA ("pUDR*") plasmids and the plasmid template used for the amplification of the backbone containing the selection marker with primer 6005 (Mans *et al.* 2015). The two fragments were combined using Gibson assembly, followed by chemical transformation to *E. coli* for storage and plasmid propagation (**Table 2**).

Plasmids containing the marker genes to complement the uracil, leucine, histidine and tryptophan auxotrophies were assembled *in vitro*, using Gibson assembly of a plasmid backbone containing the *URA3* marker and a 2µm origin of replication, with three fragments containing the *TRP1*, *LEU2* and *HIS3* marker genes. All fragments were obtained using Phusion Polymerase. The plasmid backbone was amplified from YepLDH#1 (Van Maris *et al.* 2004b) with primers 8996 & 9000 (all primers are listed in **Table S2**) and the *LEU2* fragment was obtained from pRS425 (Christianson *et al.* 1992) with primers 5892 & 9161. The *TRP1* and *HIS3* fragments were obtained from the genomic DNA of CEN.PK113-7D with primers 5891 & 8999 and 2335 & 2336 respectively.. Correct assembly of the plasmid was confirmed via restriction analysis and PCR. The resulting plasmid (pUDE412, **Table 2**) was transformed to *E. coli* via electroporation for storage and plasmid propagation.

For the construction of the lactate dehydrogenase (*LDH*) expression plasmid (pUDE411, **Table 2**), the approach described above was used with the exception that for the plasmid backbone primers 8996 and 8997 and for the *LEU2* fragment, primers 5892 and 9160 were used instead. In addition to the *URA3* marker and 2μm origin of replication, the Phusion PCR reaction using primers 8996 and 8997 also included the lactate dehydrogenase (*LDH*) gene in the plasmid backbone. Primers 5892 & 9160 were used for the *LEU2* fragment to adapt the homologous sequences flanking the *LEU2* fragment (required for Gibson assembly) to the *LDH*-containing plasmid backbone.

Strain construction

S. cerevisiae strains were transformed as described previously (Gietz and Woods 2002). Mutants were selected on synthetic medium plates supplemented with appropriate auxotrophic requirements (Verduyn *et al.* 1992; Pronk 2002). To minimize evolutionary pressure on lactate export during strain construction, a mixture of ethanol and glycerol was used as a carbon source to lower the flux through pyruvate with the aim to prevent excessive lactate formation. Gene deletions were performed as described previously (Mans *et al.* 2015) and were confirmed by colony PCR on randomly picked colonies, using the diagnostic primers listed in **Table S2**.

Strain IMX878 was obtained via removal of pUDR002, pUDR004 and pUDR005 from IMX717 (Mans *et al.* 2015), by growing in non-selective conditions as described previously (Mans *et al.* 2015). Transformation of IMX878 with pUDR007 and pUDR008 resulted in IMX880 (IMX878 + *mch3Δ mch4Δ yil166cΔ hxt1Δ*). IMX880 was then transformed with pUDR126, resulting in IMX902 (IMX880 + *jen1Δ ady2Δ*). The removal of pUDR007, pUDR008 and pUDR126 from IMX902 resulted in IMX903, which was then transformed with pUDR151 and pUDR152, resulting in IMX945 (IMX903 + *aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ*). IMX945 was then transformed with pUDR155 and after confirmation of the deletion of *ATO2* and *ATO3*, pUDR151, pUDR152 and pUDR155 were

removed, resulting in IMX973 (IMX945 + *ato2Δ ato3Δ*). IMX973 was then transformed with pUDR169, resulting in IMX987 (IMX973 + *AQY3Δ tpo2Δ*) and transformation of IMX987 with pUDR168 and subsequent removal of both pUDR-plasmids resulted in IMX999 (IMX987 + *yro2Δ azr1Δ*). IMX999 was then transformed with pUDR170 and subsequent removal of this plasmid resulted in IMX1000 (IMX999 + *yhl008cΔ tpo3Δ*).

IMX1052 and IMX1066 were made via transformation of pUDE411 to IMX672 and IMX1000 respectively, using 200 ng of plasmid DNA in the transformation reaction. IMX1053 and IMX1067 were made via transformation of pUDE412 to IMX672 and IMX1000, respectively, using 300 ng of plasmid DNA. Transformants for all 4 strains were selected on SM plates without auxotrophic requirements and subjected to quality control as described above.

Media and cultivation

Precultures for batch cultivation in bioreactors were grown in a Bactron Anaerobic Chamber (Sheldon Manufactoring, Cornelius, OR) on synthetic medium with 20 g L^{-1} glucose and the anaerobic growth factors ergosterol (10 mg L^{-1}) and Tween80 (420 mg L^{-1}) dissolved in ethanol. Strain characterization was carried out at 30°C in anaerobic 2-L laboratory bioreactors (Applikon, Delft, the Netherlands) with a working volume of 1 L. After heat sterilization (120°C for 20 min), the synthetic medium (Verduyn *et al.* 1992) was supplemented with heat sterilized (120°C for 20 min) Antifoam Emulsion C (Sigma) to a final concentration of 0.2 g L^{-1}, heat sterilized (110°C for 20 min) glucose to a final concentration of 20 g L^{-1}, the anaerobic growth factors ergosterol (10 mg L^{-1}) and Tween80 (420 mg L^{-1}) dissolved in ethanol and vitamins (Verduyn *et al.* 1992). The culture pH was maintained at 5.0 by automated addition of 2 M KOH and were stirred at 800 rpm. To maintain anaerobic conditions, the bioreactors were sparged with 500 mL N$_2$ min^{-1} (<10 ppm O$_2$) and equipped with Norprene tubing. After the batch phase, the medium pumps were switched on, resulting in the continuous addition of 100 mL min^{-1} of synthetic medium (25 g L^{-1} glucose) to the

culture. The working volume was kept constant at 1.0 L using an effluent pump controlled by an electric level sensor, resulting in a dilution rate of 0.10 h^{-1} for the chemostat phase. The exact working volume was measured at the end of each experiment. Chemostat cultures were assumed to be in steady-state when, after five volume changes, the culture dry weight, extracellular metabolite concentrations of ethanol and glycerol and the CO_2 production rate varied by less than 2% over at least 2 additional volume changes.

Analytical methods

The optical density of cultures was monitored using a Libra S11 spectrophotometer (Biochrom, Cambridge, United Kingdom) at 660 nm. Culture dry weights were determined via filtration of samples over dry nitrocellulose filters with a pore size of 0.45 µm (Gelman laboratory, Ann Arbor, USA). Prior to filtration, the filters were dried and weighed. After filtration of the sample, the filters were washed again using demineralized water and then dried in a microwave oven for 20 min at 360 W and weighed again. Supernatant was obtained via centrifugation of the culture broth and samples for residual sugars were quenched using cold stainless-steel beads (Mashego *et al.* 2003). HPLC-analysis of the supernatant and residual sugar samples was performed as described previously (De Kok *et al.* 2011) and ethanol concentrations were corrected for ethanol evaporation (Guadalupe-Medina *et al.* 2010). Off-gas was cooled in a condenser and dried with a Perma Pure Dryer (Permapure, Lakewood, NJ) before CO_2 concentrations were measured with a NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO). To determine intracellular lactate concentrations, 50 mL of culture broth was spun down and the supernatant was discarded. The pellet was then resuspended in demineralized water and boiled for 20 minutes at 100°C. The supernatant from the boiled sample was collected via centrifugation and analysed via HPLC. To calculate the intracellular concentrations, the sample volume, pellet volume and the volume of demineralized water were quantified based on weight, assuming 1 g = 1 mL. To determine the biomass weight in the pellet and the extracellular concentration of lactate, a second sample was

taken at the same time point, on which dry weight determination and HPLC-analysis were performed as described previously. For conversion of g lactate (g dry cell weight)$^{-1}$ to estimated intracellular lactate concentrations, a cell volume of 2 mL (g dry cell weight)$^{-1}$ was assumed (Kresnowati, Groothuizen and Van Winden 2007).

Determination of *in vitro* lactate dehydrogenase activity

For enzymatic activity measurements, culture samples corresponding to 62.5 mg dry weight were harvested from steady-state chemostat cultures. Cell extracts were prepared by sonication and centrifugation (Postma *et al.* 1989). Lactate dehydrogenase activity was measured at 30°C with a reaction mixture consisting of 2 mM MnCl$_2$, 1 mM fructose-1,6-diphosphate and 0.15 mM NADH in 0.1 M imidazole at pH 6.5. Reactions were started by addition of 10 mM potassium pyruvate (Van Maris *et al.* 2004b).

Results

Identification of candidate genes for knock-out study

The currently available knowledge on lactate export by *S. cerevisiae* suggests the involvement of multiple transporters in this process. To try and capture multiple redundant transporters, a set of candidate membrane-protein-encoding genes was defined and deleted in a single *S. cerevisiae* strain. A set of 25 candidate lactate export genes was compiled based on the following criteria (**Table 3**): (i) a proposed involvement in lactate transport or (ii) a proposed involvement in lactate tolerance or (iii) a proposed role in carboxylic acid transport or (iv) carboxylic acid resistance.

First, genes that were previously shown to be involved in lactate uptake were selected (**Table 3**). *JEN1* and *ADY2* have both been described to be involved in the uptake of lactate (De Kok *et al.* 2012; Pacheco *et al.* 2012). *ATO2* and *ATO3* were also selected, based on the sequence similarities of their encoded proteins to Ady2 (protein BLAST *e*-values of ≤ 10^{-164} and ≤ 10^{-36}

respectively). Subsequently, 4 transporter genes (*HXT1*, *ITR1*, *PDR12* and *AQY1*) were selected based on a single-gene deletion screening in which their deletion was reported to reduce lactate production by >50% (Hirasawa *et al.* 2013). Aqua(glycero)porins have been shown to transport lactate in several organisms (Tsukaguchi *et al.* 1998; Choi and Roberts 2007; Bienert *et al.* 2013) and large strain-to-strain variations of aqua(glycero)porin genes occur in yeasts (Sabir, Loureiro-Dias and Prista 2016). Therefore, the aqua(glycero)porins *FPS1*, *AQY1*, *AQY2*, *AQY3* and YLL053C (contiguous with *AQY2*) (Coury *et al.* 1999; Mollapour and Piper 2007; Sabir, Loureiro-Dias and Prista 2016) were selected for gene deletion. Another group of transporter-encoding genes, consisting of the Haa1p-regulated genes *TPO2*, *TPO3* and *YRO2* (**Table 3**), was selected based on their involvement in resistance to lactic acid stress at low pH (Abbott *et al.* 2008).

As a third category, genes were selected that have not directly been implicated in lactate transport or tolerance, but that have been linked to transport of other carboxylic acids. *MCH1, MCH2, MCH3, MCH4, MCH5* were selected based on their similarities with the mammalian monocarboxylate transporters (MCT), some of which are known to transport lactate (Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010). *THI73* and YIL166C are members of the anion:cation symporter subfamily (all members are putative weak-acid permeases) with a suggested plasma membrane localization and were selected based on their unknown substrate specificity (Paulsen *et al.* 1998; Mojzita and Hohmann 2006; Hellborg *et al.* 2008). Additionally, the putative acetic-acid transporter encoding gene YHL008C (Paulsen *et al.* 1998) was selected for deletion. As a last category, *AQR1* and AZR1 were selected in view of their reported involvement in tolerance of *S. cerevisiae* to short-chain monocarboxylic acids (Tenreiro *et al.* 2000, 2002; Bauer *et al.* 2003; Velasco *et al.* 2004).

Deletion of 25 transporter genes

To investigate whether any of the selected candidate genes are involved in the export of lactate, the complete set of 25 genes was deleted in the CEN.PK yeast background. Strain IMX717, which contains deletions in six of the candidate genes (*MCH1*, *MCH2*, *MCH5*, *AQY1*, *ITR1* and *PDR12;* Mans *et al.* 2015), was used as a starting point to construct a single strain that contained 25 transporter gene deletions. The deletion of all 25 transporter genes in strain IMX1000 was investigated by colony PCR, using primers that bind outside each of the deleted open reading frames (**Figure 2)**. As a control, the same PCR reactions were performed on the DNA of the reference strain CEN.PK113-7D in which all 25 genes were intact according to a published whole-genome sequence (Nijkamp *et al.* 2012). Size analysis of the PCR products on gel confirmed successful deletion of all 25 candidate genes in IMX1000 (**Figure 2, Table S3**).

Lactate formation in anaerobic bioreactor batch cultures

To investigate whether the combined deletion of the 25 membrane-protein encoding candidate genes had a significant effect on the lactate production rate, an expression cassette for a lactate dehydrogenase gene from *Lactobacillus casei* (*LcLDH*), which previously demonstrated high activities in CEN.PK *S. cerevisiae* strains (Van Maris *et al.* 2004b), was introduced on a multi-copy plasmid, resulting in strains IMX1052 (reference + *LcLDH*) and IMX1066 (25 deletions + *LcLDH*). Lactate-dehydrogenase activities in cell extracts of these two strains were not significantly different (5.6 ± 0.3 and 5.6 ± 0.1 μmol min^{-1} mg of protein^{-1} for IMX1052 and IMX1066 respectively). Since a homolactic strain with abolished lactate transport may be inhibited by intracellular accumulation of lactate, the alcoholic fermentation pathway in these strains was left intact. This approach allows a distribution of pyruvate between the formation of lactate and ethanol (**Figure 1**), resulting in the formation of both products (Dequin and Barre 1994; Brambilla *et al.* 1999; Hirasawa *et al.* 2013). Another advantage of leaving the alcoholic fermentation pathway intact is that it allows for net

conservation of free-energy in the form of ATP from substrate-level phosphorylation, thereby enabling anaerobic growth.

Both strains were grown in pH-controlled anaerobic batch bioreactors and formation of ethanol and lactate were monitored (**Figure 3**). The pH was maintained at 5.0, as under these conditions ATP-dependent export of lactic acid was demonstrated previously (Van Maris *et al.* 2004b). *LDH* overexpression in the reference strain IMX1052 indeed resulted in formation of lactate and ethanol as the major fermentation products. Upon glucose depletion (34.5 h), 152.4 ± 0.5 mM ethanol and 14.3 ± 0.0 mM lactate (ratio 11 : 1) had been formed. Under identical conditions, the 25-deletion strain IMX1066 accumulated 156.7 ± 0.3 mM ethanol and 7.7 ± 0.2 mM lactate (ratio 20 : 1) after 43.5 h (**Figure 3**). The almost twofold reduction in lactate formation could indicate a reduced capacity for lactate export, leading to higher intracellular lactate concentrations which might in turn favour the formation of ethanol from pyruvate in strain IMX1066. Not only the lactate-ethanol ratio, but also the biomass specific lactate ($0.67 ± 0.01$ mmol (g of biomass)$^{-1}$ h^{-1}) and ethanol production rate ($11.9 ± 0.0$ mmol (g of biomass)$^{-1}$ h^{-1}) of IMX1066 were significantly lower than that of the reference strain ($2.38 ± 0.02$ mmol lacate and $22.7 ± 0.6$ mmol ethanol (g of biomass)$^{-1}$ h^{-1}). However, also the specific growth rate of IMX1066 was substantially lower ($0.15 ± 0.00$ h^{-1}) than that of the isogenic reference strain IMX1052 ($0.25 ± 0.00$ h^{-1}). Therefore, an effect of specific growth rate on the distribution of pyruvate over ethanol and lactate could not be excluded. The lower specific growth rate of the 25-deletion strain IMX1066 was not related to the presence of lactic acid or overexpression of *LDH* in this genetic background, as the isogenic 25-deletion control strain IMX1067 (25 deletions, no *LcLDH*) grew with the same specific rate of $0.16 ± 0.00$ h^{-1} under identical conditions (**Figure 3**).

In contrast to batch cultivation, chemostat cultivation enables comparison between strains at identical, predefined specific growth rates. To eliminate a possible impact of specific growth rate

on the comparison of lactate production in the 25-deletion and reference strains, a quantitative comparison of lactate and ethanol production between IMX1052 and IMX1066 was made in anaerobic glucose-limited chemostat cultures grown at a fixed dilution rate of 0.10 h^{-1} (**Table 4**). At a specific growth rate of 0.10 h^{-1}, both strains showed identical biomass-specific rates of ethanol and lactate production. Moreover, no significant differences were observed in any of the other measured parameters (**Table 4**). In line with the hypothesis that at low growth rates, the formation of ethanol is favoured over the formation of lactate, a distribution of 20 ethanol : 1 lactate was observed, which is similar to the distribution found for IMX1066 in anaerobic batch cultures at a specific growth rate of 0.15 h^{-1} (**Figure 3**). In addition, intracellular lactate concentrations in the two strains were not significantly different (**Table 4**).

Discussion

The present study, in which 25 membrane-protein-encoding genes were inactivated in a single *S. cerevisiae* strain by multiple cycles of CRISPR/Cas9-mediated gene deletion, illustrates the potential of this genome-editing technique for functional analysis. In total, 8 transformations and 5 marker recycling steps were used for the construction of IMX1000 (25 deletions) from the parental strain IMX672 (Mans *et al.* 2015). Since, at the outset of the strain construction efforts, lethality of some combinations of mutations could not be excluded, the number of simultaneous deletions in most transformations was limited to 2 or 4. This decision implied that the full potential of the combination of Cas9 and gRNA-plasmids used in this study (at least 6 simultaneous mutations) was not used. Nevertheless and despite the relatively low specific growth rate of *S. cerevisiae* on ethanol (Van Dijken *et al.* 2000), the combined introduction of 25 gene deletions could be completed in 90 ± 5 days of strain construction (3.6 days per deletion). In the absence of laboratory automation, this lowers strain construction times by more than twofold compared to Cre-*LoxP* (9 days per deletion)

or Green monster (9 days per deletion) strategies for genome editing (Delneri *et al.* 2000; Suzuki *et al.* 2011; Bao *et al.* 2015).

In addition to insights into the mechanism of lactate transport, the experiments performed in this study also provide insight into (homo)lactic acid fermentation in *S. cerevisiae*. The observed specific lactate production rate of strain IMX1052 (2.4 ± 0.0 mmol (g of biomass)$^{-1}$ h^{-1}) is substantially higher than reported for a homolactic pyruvate-decarboxylase-negative *S. cerevisiae* incubated under anaerobic conditions (0.8 mmol g of biomass^{-1} h^{-1}, van Maris *et al.* 2004b). Under anaerobiosis, homolactic *S. cerevisiae* strains have been shown to rapidly deplete intracellular ATP, resulting in extremely low adenylate energy charges within 30 min after a switch to anaerobic conditions (Abbott *et al.* 2009). The high anaerobic lactate production rate observed in cultures of strain IMX1052 therefore probably reflects availability of additional ATP from alcoholic fermentation (**Figure 1**).

The lower growth rate and the lower summed biomass-specific fluxes of ethanol and lactate of strain IMX1066 in anaerobic batch cultures compared to the reference strain IMX1052 indicate a lower glycolytic flux (required to provide ATP and metabolites for biomass formation) relative to the reference strain. It has previously been shown that, in *S. cerevisiae*, lower glycolytic fluxes correlate with lower intracellular concentrations of glycolytic intermediates (Canelas *et al.* 2010). Considering the lower K_m of *S. cerevisiae* pyruvate decarboxylase for pyruvate (0.65 mM, (Kresze and Ronft 1981)) compared to *L. casei* lactate dehydrogenase (1.0 mM, (Gordon and Doelle 1976)), lower intracellular pyruvate concentrations at lower glycolytic flux, might favour alcoholic fermentation over lactate fermentation (**Figure 1**). This hypothesis is consistent with the observation that both strains exhibited a similar flux distribution in chemostat cultures grown at identical growth rates of 0.10 h^{-1} (**Table 4**).

The absence of a significant impact of the 25 deletions on lactate production rates in the anaerobic, glucose-limited chemostats could, in theory, reflect a situation in which a reduced lactate export capacity was exactly compensated for by an increased thermodynamic driving force or by increased saturation of exporters with intracellular substrate. In both cases, this would require an increased intracellular lactate concentration. Since the 25-deletion strain did not show an increased intracellular lactate concentration (**Table 4**), the experimental data support the conclusion that deletion of these 25 (putative) transporter genes did not significantly affect lactate export capacity in IMX1066 under the experimental conditions.

The question remains which transporter(s) or transport mechanisms are responsible for lactate export in strain IMX1066. The observation that a mutant lacking both *JEN1* and *ADY2* is unable to grow on lactate (De Kok *et al.* 2012) and the conclusion that lactate export is in all likelihood energy coupled (Van Maris *et al.* 2004b; Abbott *et al.* 2009), suggest a unidirectional (export only) transport mechanisms under the conditions studied (extracellular pH 5.0). These considerations leave three plausible mechanisms for lactate transport: i) primary transport catalysed by an ABC-transporter, ii) antiport of lactic acid with a proton or other ion, iii) (facilitated) diffusion of the lactate anion and extrusion of the accompanying proton via the H^+-ATPase (Van Maris *et al.* 2004a).

PDR12, encoding the only ABC-transporter with a confirmed role in transport of the weak-acid anions (Piper *et al.* 1998; Holyoak *et al.* 1999), was deleted in this study. Of the remaining 11 ABC transporters with transmembrane domains and localisation in the plasma membrane (Kerr 2004; Zhao *et al.* 2004, 2006; Paumi *et al.* 2009), only Pdr5, Pdr10 and Yor1 are known to be induced by more lipophilic weak acids, although a transport function of these proteins has not been unequivocally demonstrated (Mira, Teixeira and Sá-Correia 2010). Based on the measured intracellular lactate concentration (**Table 4**), antiport of lactic acid with an ion (such as H^+ or Na^+) and subsequent extrusion of the ion at the expense of 1 ATP via a P-type ATPase, is a

thermodynamically feasible transport mechanism. Lactic acid/proton-antiport would also be in agreement with the observation that combined deletion of *JEN1* and *ADY2* prevented growth on 55 mM lactate at an extracellular pH of 5.0, which would only allow a maximum achievable intracellular lactic-acid concentration of 0.2 mM (assuming a thermodynamic equilibrium and a constant proton-motive-force of -0,15 V and intracellular pH of 7.0; Van Maris *et al.* 2004a). The measured intra- and extracellular lactate concentrations (**Table 4**) in combination with a negative membrane potential, result in a driving force for (facilitated) diffusion of the lactate anion.

Ion channels in the plasma membrane of *S. cerevisiae* have been reported to facilitate the diffusion of small cations such as potassium, metals and ammonium (Ketchum *et al.* 1995; Marini *et al.* 1997; Eide 1998). To the best of our knowledge, no such mechanisms for anions have been described. Although free diffusion of charged molecules such as the anion thiocyanate (SCN$^-$) and the cation tetraphenylphosphonium (TPP) has been described, this is facilitated by their hydrophobicity and, in the case of TPP, charge delocalization by phenyl residues (Gutknecht and Walter 1982; Skulachev 2007; Trendeleva *et al.* 2012). Despite the generally accepted assumption that anions of polar carboxylic acids do not freely diffuse over the cell membrane (Cássio, Leao and Van Uden 1987; Lambert and Stratford 1999), it seems relevant to experimentally test this assumption in *in vitro* experiments.

The Yeast Transport Protein database (YTPdb) predicts a total number of 302 membrane transporter proteins in *S. cerevisiae* of which 139 have a predicted localization to the plasma membrane, 106 have a predicted localization to internal membrane and for the remaining 57 transporters the localization is unknown (Brohée *et al.* 2010). Of the genes deleted in IMX1000, 23 are classified as transporters according to YTPbd, 13 with a predicted plasma membrane localization, 3 with a predicted localization to internal membrane and 7 with unknown localization. Additionally, IMX1000 contains an integrated copy of Cas9 in the *CAN1* locus, thereby inactivating

the plasma-membrane arginine transporter. The 25-deletion strain constructed in this study contains deletions of complete functional transporter groups, such as water transport (mediated by *AQY1*, *AQY2*, *AQY3* and *FPS1*, for an extensive review see (Ahmadpour *et al.* 2014)), glycerol export (mediated by *FPS1*) and all known carboxylic acid transporters (*JEN1*, *ADY2*, *FPS1* and *PDR12*) and provides a convenient platform for further studies. The search for the lactic-acid export mechanisms in *S. cerevisiae* might benefit from additional CRISPR/Cas9-based deletion of the remaining genes belonging to the 12 spanner drug:H$^+$-antiporters family (Sá-Correia and Tenreiro 2002; Mira, Teixeira and Sá-Correia 2010) and the remaining plasma-membrane located ABC-transporters, other than Pdr12. Systematic deletion of all members of the five transporter-encoding gene families that are non-essential for growth in synthetic laboratory media, could ultimately yield a 'minimal transportome yeast'. As proposed for other 'minimalist yeast' genome configurations and already demonstrated for the HXT-null and PDR-null strains (Wieczorke *et al.* 1999; Suzuki *et al.* 2011), such a strain could be highly valuable for functional analysis of (heterologous) genes in *S. cerevisiae* (Oliver 1996). Additionally, a strain in which non-essential transporters have been systematically removed will be highly informative for strategies aimed at preventing protein crowding in membranes (Engelman 2005; Coskun and Simons 2010; Chavent, Duncan and Sansom 2016) of metabolically engineered yeast strains.

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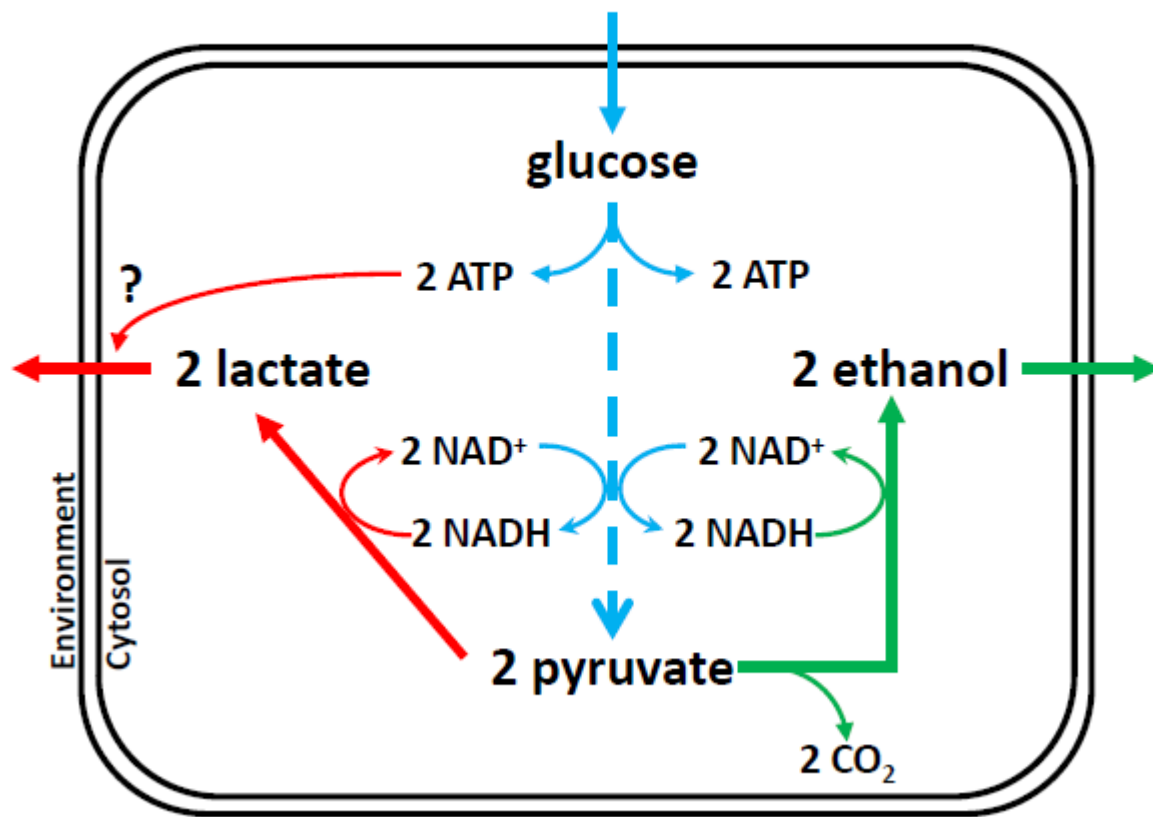
Figure 1 – Schematic representation of fermentation of glucose to ethanol or lactate. Uptake of

glucose proceeds via facilitated diffusion and subsequent conversion by the Embden-Meyerhof-

Parnas (EMP) pathway leads to the formation of 2 ATP, 2 NADH and 2 pyruvate (blue). Conversion

of 2 pyruvate to 2 ethanol via pyruvate decarboxylase and alcohol dehydrogenase activity leads to

the re-oxidation of 2 NADH to 2 NAD^{+} and diffusion of ethanol out of the cell is energy-neutral

(green). In homolactic engineered *S. cerevisiae* strains, the conversion of 2 pyruvate to 2 lactate by

lactate dehydrogenase activity also leads to the re-oxidation of 2 NADH to 2 NAD^{+}. However,

inability of homolactic strains to grow anaerobically indicates that the ATP formed by the EMP-

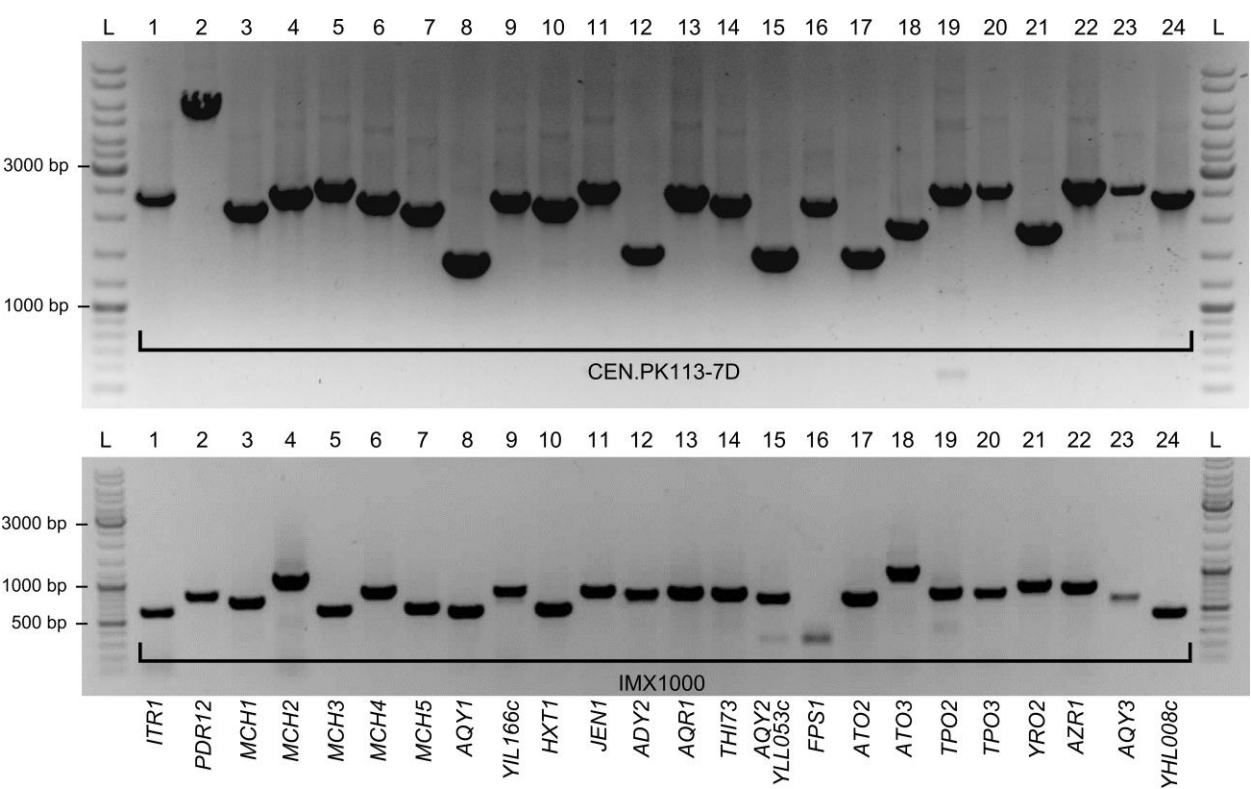pathway is used for lactate export (red), thereby leaving no ATP for anaerobic growth or cellular maintenance.

Figure 2 – Diagnostic gel for verification of the correct deletion of 25 membrane-protein-encoding genes in *S. cerevisiae* strain IMX1000. The left and right lanes(L) contain GeneRuler DNA Ladder Mix. Lanes 1-24 were loaded with PCR fragments amplified from genomic DNA of *S. cerevisiae* strains CEN.PK113-7D (reference) or IMX1000 with oligonucleotide primers that bind outside of the target ORF. Lane 15 shows PCR fragments obtained with primers that bind outside both *AQY2* and YLL053C (contiguous with *AQY2*) and which were used to verify both deletions simultaneously. Primers used for the PCR and expected band sizes are listed in **Table S3**.
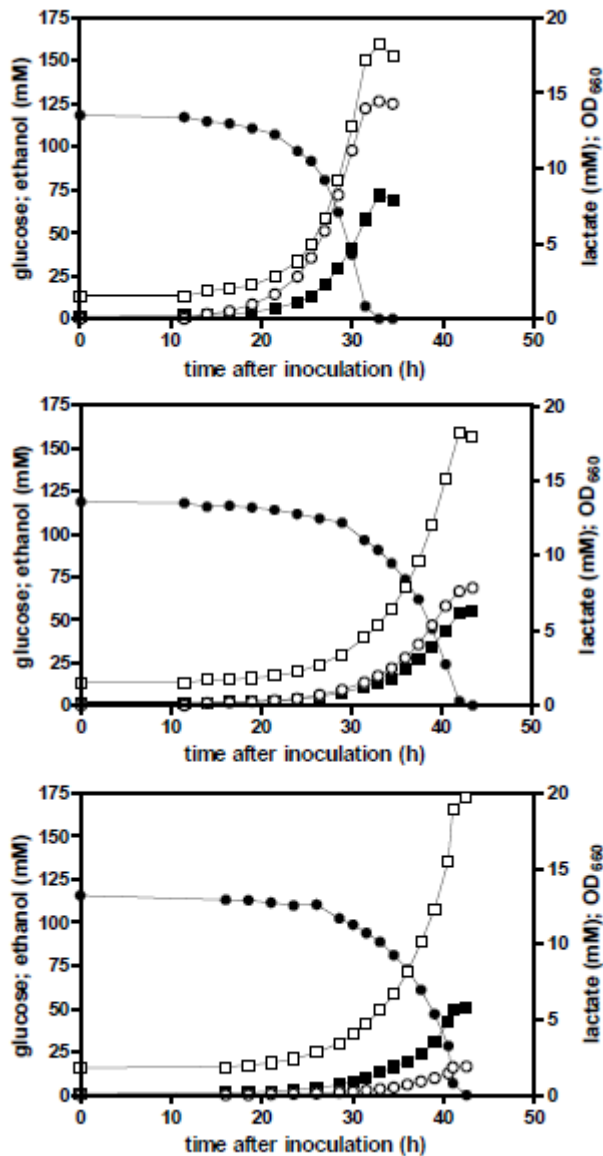
Figure 3 – Concentrations of biomass and metabolites in anaerobic batch cultures of *S. cerevisiae* IMX1052 (top, reference strain expressing *LcLDH*), IMX1066 (middle, 25 deletions in membrane-protein-encoding genes and expressing *LcLDH*) and IMX1067 (bottom, 25 deletions in membrane-protein-encoding genes without expression of *LcLDH*) when grown in anaerobic bioreactors in on a synthetic medium containing 20 g L⁻¹ glucose at 30°C and at pH 5.0. Symbols: ●, glucose; ■, optical density at 660 nm; ○, lactate; □, ethanol. Each graph shows representative data from one of two independent replicates.

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Name	Relevant genotype	Parental strain	Origin
CEN.PK113-7D	*MATα URA3 TRP1 LEU2 HIS3*		P. Kötter
IMX672	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2*	CEN.PK2-1C	(Mans *et al.* 2015)
IMX717	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ* pUDR002 (2μm ampR *TRP1* gRNA-*MCH1* gRNA-*MCH2*) pUDR004 (2μm ampR *HIS3* gRNA-*MCH5* gRNA-*AQY1*) pUDR005 (2μm ampR *URA3* gRNA-*ITR1* gRNA-*PDR12*)	IMX672	(Mans *et al.* 2015)
IMX878	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ*	IMX717	This study
IMX880	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ* pUDR007 (2μm ampR *URA3* gRNA-*MCH3* gRNA-*MCH4*) pUDR008 (2μm ampR *HIS3* gRNA-YIL166C gRNA-*HXT1*)	IMX878	This study
IMX902	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ* pUDR007 (2μm ampR *URA3* gRNA-*MCH3* gRNA-*MCH4*) pUDR008 (2μm ampR *HIS3* gRNA-YLL053C gRNA-*HXT1*) pUDR126 (2μm ampR *KlLEU2* gRNA-*JEN1* gRNA-*ADY2*)	IMX880	This study
IMX903	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ*	IMX902	This study
IMX945	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ* pUDR151 (2μm ampR *KlLEU2* gRNA-*AQR2* gRNA-*THI73*) pUDR152 (2μm ampR *HIS3* gRNA-*FPS1* gRNA-*AQY2*-YLL053C)	IMX903	This study
IMX973	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ*	IMX945	This study
IMX987	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ* pUDR169 (2μm ampR *HIS3* gRNA-*AQY3* gRNA-*TPO2*)	IMX973	This study
IMX999	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ*	IMX987	This study

IMX1000	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ yhl008cΔ tpo3Δ*	IMX999	This study
IMX1052	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2* pUDE411 (2μm ampR *URA3 TRP1 LEU2 HIS3 LcLDH*)	IMX672	This study
IMX1053	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2* pUDE412 (2μm ampR *URA3 TRP1 LEU2 HIS3*)	IMX672	This study
IMX1066	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ yhl008cΔ tpo3Δ* pUDE411 (2μm ampR *URA3 TRP1 LEU2 HIS3 LcLDH*)	IMX1000	This study
IMX1067	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ yhl008cΔ tpo3Δ* pUDE412 (2μm ampR *URA3 TRP1 LEU2 HIS3*)	IMX1000	This study

Table 2. Plasmids used in this study.

Name	Relevant characteristics	Origin
pROS10	2μm ampR *URA3* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	(Mans *et al.* 2015)
pROS14	2μm ampR *KlLEU2* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	(Mans *et al.* 2015)
pROS16	2μm ampR *HIS3* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	(Mans *et al.* 2015)
pUDR007	2μm ampR *URA3* gRNA-*MCH3* gRNA-*MCH4*	This study
pUDR008	2μm ampR *HIS3* gRNA-YLL053C gRNA-*HXT1*	This study
pUDR126	2μm ampR *KlLEU2* gRNA-*JEN1* gRNA-*ADY2*	This study
pUDR151	2μm ampR *KlLEU2* gRNA-*AQR2* gRNA-*THI73*	This study
pUDR152	2μm ampR *HIS3* gRNA-*FPS1* gRNA-*AQY2*-YLL053C	This study
pUDR155	2μm ampR *URA3* gRNA-*ATO2* gRNA-*ATO3*	This study
pUDR168	2μm ampR *URA3* gRNA-*YRO2* gRNA-*AZR1*	This study
pUDR169	2μm ampR *HIS3* gRNA-*AQY3* gRNA-*TPO2*	This study
pUDR170	2μm ampR *KlLEU2* gRNA-YHL008C gRNA-*TPO3*	This study
pUDE411	2μm ampR *URA3 TRP1 LEU2 HIS3 LcLDH*	This study
pUDE412	2μm ampR *URA3 TRP1 LEU2 HIS3*	This study

pRS425		Template for amplification of *LEU2*	(Christianson *et al.* 1992)
YepLDH#1		Template for amplification of 2μm ampR *URA3 LcLDH*	(van Maris *et al.* 2004b)
		Template for amplification of 2μm ampR *URA3*	

Table 3. embrane-protein-encoding *S. cerevisiae* genes, included in a systematic deletion study aimed at identifying membrane transporters involved in lactate export and motivation for their selection.

Gene	Std. name	Motivation for selection	Reference(s)
Group i) genes (potentially) involved in lactate transport			
JEN1	YKL217W	Known lactate transporter	(de Kok *et al.* 2012; Pacheco *et al.* 2012)
ADY2	YCR010C	Known lactate transporter	(de Kok *et al.* 2012; Pacheco *et al.* 2012)
ATO2	YNR002C	Similar to Ady2 (BLASTp e-value of $\leq 10^{-164}$)	This study
ATO3	YDR384C	Similar to Ady2 (BLASTp e-value of $\leq 10^{-36}$)	This study
HXT1	YHR094C	Target from knock-out screening	(Hirasawa *et al.* 2013)
ITR1	YDR497C	Target from knock-out screening	(Hirasawa *et al.* 2013)
PDR12	YPL058C	Target from knock-out screening	(Hirasawa *et al.* 2013)
AQY1	YPR192W	Aqua(glycero)porin & target from knock-out screening	(Coury *et al.* 1999; Sabir, Loureiro-Dias and Prista 2016)
FPS1	YLL043W	Aqua(glycero)porin involved in acetic acid uptake	(Mollapour and Piper 2007)
AQY2	YLL052C	Aqua(glycero)porin	(Coury *et al.* 1999; Sabir, Loureiro-Dias and Prista 2016)
AQY3	YFL054C	Aqua(glycero)porin	(Coury *et al.* 1999; Sabir, Loureiro-Dias and Prista 2016)
	YLL053C	Aqua(glycero)porin	(Coury *et al.* 1999; Sabir, Loureiro-Dias and Prista 2016)
Group ii) genes (potentially) involved in lactic acid resistance			
TPO2	YGR138C	Expression controlled by the Haa1p transcriptional regulator	(Abbott *et al.* 2009)

TPO3	YPR156C	Expression controlled by the Haa1p transcriptional regulator	(Abbott *et al.* 2009)
YRO2	YBR054W	Expression controlled by the Haa1p transcriptional regulator	(Abbott *et al.* 2009)

Group iii) genes (potentially) involved in transport of other carboxylic acids

MCH1	YDL054C	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
MCH2	YKL221W	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
MCH3	YNL125C	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
MCH4	YOL119C	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
MCH5	YOR306C	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
THI73	YLR004C	Plasma membrane anion:cation symporter with unknown substrate specificity	(Paulsen *et al.* 1998; Mojzita and Hohmann 2006; Hellborg *et al.* 2008)
	YIL166C	Plasma membrane anion:cation symporter with unknown substrate specificity	(Paulsen *et al.* 1998; Mojzita and Hohmann 2006; Hellborg *et al.* 2008)
	YHL008C	Putative acetic acid transporter	(Paulsen *et al.* 1998)

Group iv) genes (potentially) involved in resistance to other carboxylic acids

AQR1	YNL065W	Resistance to short-chain monocarboxylic acids	(Tenreiro *et al.* 2002; Velasco *et al.* 2004)
AZR1	YGR224W	Resistance to acetic acid	(Tenreiro *et al.* 2000; Bauer *et al.* 2003)

Table 4

Physiological parameters of the *L. casei* lactate dehydrogenase (*LcLDH*)-expressing reference *S. cerevisiae* strain IMX1052 and the *LcLDH*-expressing strain IMX1066 (25 deletions in selection membrane-protein-encoding genes) in anaerobic glucose-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1}. Averages and mean deviations were obtained from independent duplicate experiments of each strain.

Strain	IMX1052	IMX1066
Relevant genotype	Isogenic reference + *LcLDH*	25 transporters deleted + *LcLDH*
Dilution rate	0.10 ± 0.00	0.10 ± 0.00
$q_{glucose}$ (mmol g biomass^{-1} h^{-1})	6.8 ± 0.1	6.6 ± 0.2
q_{CO2} (mmol g biomass^{-1} h^{-1})	10.5 ± 0.2	10.2 ± 0.1
$q_{ethanol}$ (mmol g biomass^{-1} h^{-1})	10.0 ± 0.5	10.0 ± 0.2
$q_{lactate}$ (mmol g biomass^{-1} h^{-1})	0.48 ± 0.02	0.47 ± 0.01
$q_{glycerol}$ (mmol g biomass^{-1} h^{-1})	0.93 ± 0.00	0.85 ± 0.02
$q_{pyruvate}$ (mmol g biomass^{-1} h^{-1})	0.01 ± 0.00	0.01 ± 0.00
$q_{acetate}$ (mmol g biomass^{-1} h^{-1})	0.04 ± 0.00	0.03 ± 0.00
Residual glucose (mmol L^{-1})	0.63 ± 0.02	0.54 ± 0.08
Intracellular lactate (mmol L^{-1})	20.6 ± 0.8	21.4 ± 1.4
Extracellular lactate (mmol L^{-1})	10.5 ± 0.1	10.6 ± 0.1