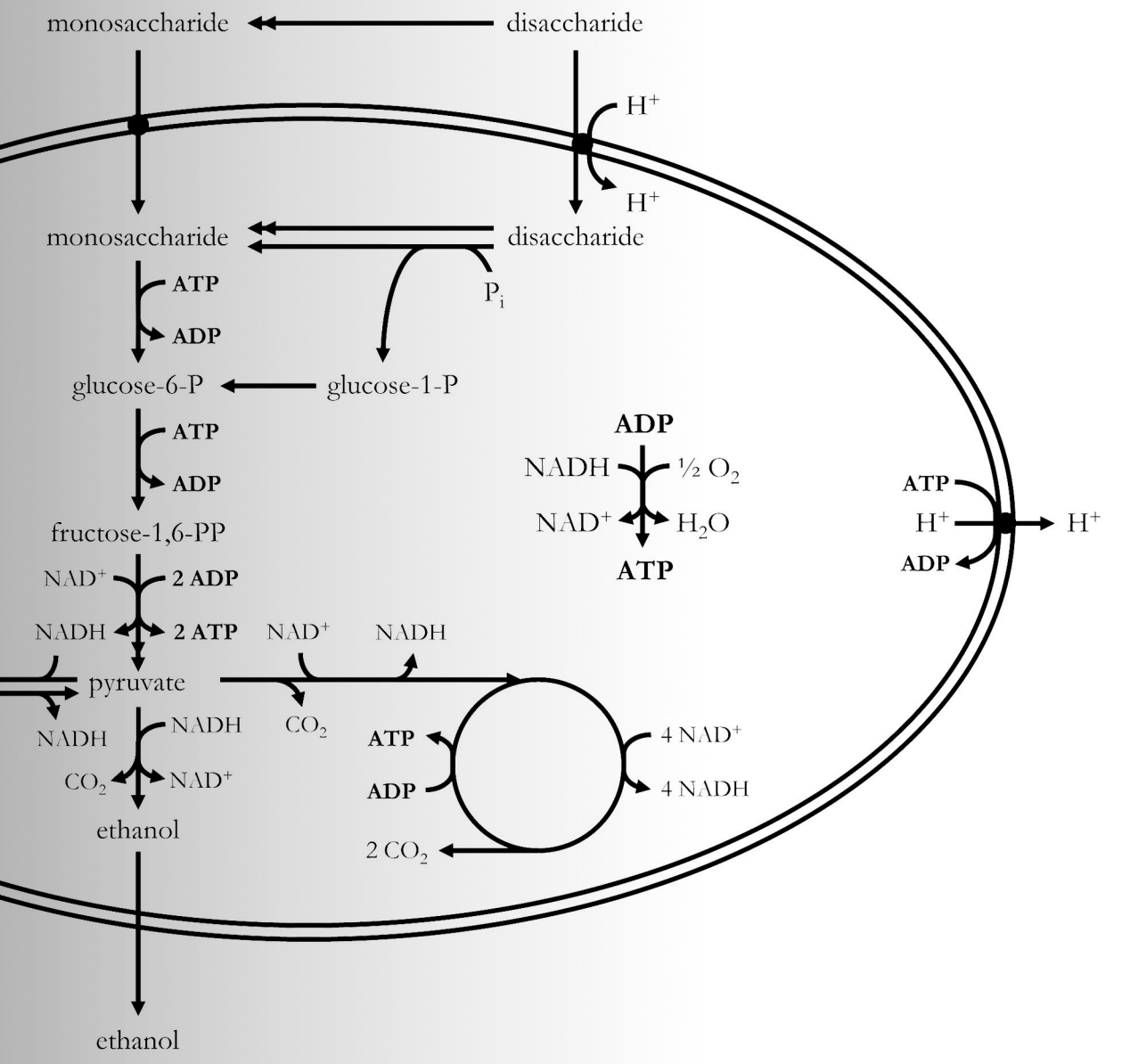
Metabolic engineering of free-energy (ATP) conserving reactions in *Saccharomyces cerevisiae*

Stefan de Kok



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Metabolic engineering of free-energy (ATP) conserving reactions in *Saccharomyces cerevisiae*

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Stefan DE KOK

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Prof. dr. J.T. Pronk

Copromotor:
Dr. ir. A.J.A. van Maris

Samenstelling promotiecommissie:

Rector Magnificus	voorzitter
Prof. dr. J.T. Pronk	Technische Universiteit Delft, promotor
Dr. ir. A.J.A. van Maris	Technische Universiteit Delft, copromotor
Prof. dr. ir. J.J. Heijnen	Technische Universiteit Delft
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Dr. R.A. Weusthuis	Wageningen University
Prof. dr. P.D.E.M. Verhaert	Technische Universiteit Delft, reservelid

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The cover shows a graphical abstract of the work presented in this thesis.

Contents

Chapter 1

Energy coupling in *Saccharomyces cerevisiae*: selected opportunities for metabolic engineering

Stefan de Kok, Barbara U. Kozak, Jack T. Pronk and Antonius J.A. van Maris

Manuscript in preparation

Abstract

Free-energy (ATP) conservation during product formation is crucial for the maximum product yield that can be obtained, but often overlooked in metabolic engineering strategies. Product pathways that do not yield ATP or even demand input of free energy (ATP) require an additional pathway to supply the ATP needed for product formation, cellular maintenance and/or growth. On the other hand, product pathways with a high ATP yield may result in excess biomass formation at the expense of the product yield. This mini-review discusses the importance of the ATP yield for product formation and presents several opportunities for engineering free-energy (ATP) conservation, with a focus on sugar-based product formation by *Saccharomyces cerevisiae*. These engineering opportunities are not limited to the metabolic flexibility within *S. cerevisiae* itself, but also expression of heterologous reactions will be taken into account. As such, the diversity in microbial sugar uptake and phosphorylation mechanisms, carboxylation reactions, product export and the flexibility of oxidative phosphorylation via the respiratory chain and H^+-ATP synthase can be used to increase or decrease free-energy (ATP) conservation. For product pathways with a negative, zero or too high ATP yield, analysis and metabolic engineering of the ATP yield of product formation will provide a promising strategy to increase the product yield and simplify process conditions.

Introduction

Accelerated by spectacular developments in recombinant-DNA technology, DNA sequencing, DNA synthesis, functional genomics, systems biology and high-throughput experimentation, metabolic engineering increasingly provides viable alternatives for petrochemistry-based production of chemicals. Products of engineered microorganisms range from pharmaceuticals (e.g. the anti-malarial precursor artemisinic acid and hydrocortisone) to bulk chemicals (e.g. 1,3-propanediol) and fuels (e.g. isobutanol) [5, 50, 58, 73].

Especially for microbial processes aimed at the production of commodity chemicals, costs of the carbon substrate strongly influence process economy. For instance, sugar costs have been estimated to make up 70% of the cost price of bioethanol [47]. Process economy therefore provides a strong incentive to push product yields of engineered microorganisms towards their thermodynamic and stoichiometric limits by optimization of product pathways.

Once pathways that lead to byproduct formation have been eliminated, several aspects of microbial metabolic networks determine the maximum feasible product yield on substrate. Firstly, the biochemical pathways operating in an industrial microorganism can constrain conversion coefficients for carbon and other elements. For example, if conversion of glucose to acetyl-CoA, a key intermediate of lipid synthesis, exclusively proceeds via a $C_3 \rightarrow C_2$ decarboxylation step (e.g. via pyruvate dehydrogenase), then this imposes an upper limit to the lipid yield on glucose of 0.667 $Cmol \cdot Cmol^{-1}$. Secondly, product yields can be constrained by the need to balance oxidation and reduction of redox cofactors (e.g. $NAD(P)^+$ and NAD(P)(H)) [85]. The impact of redox balances on product yields is illustrated by comparison of different metabolic pathways for anaerobic production of ethanol from xylose and arabinose [12, 76]. This review focuses on a third important factor: conservation of free energy (ATP) during product formation.

Product pathways with a positive net ATP yield provide microorganisms with free energy for growth and maintenance processes. However, during industrial production of chemicals, excess microbial biomass constitutes an undesirable byproduct, whose formation goes at the expense of the product yield. Optimal product yields will therefore be reached at low, but positive yields of ATP in the product pathway, which are sufficient to maintain cellular performance but do not allow for unrestricted growth. The validity of

this statement is illustrated by a comparison of the alcoholic fermentation of glucose to ethanol by wild-type strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis*. In *S. cerevisiae*, alcoholic fermentation via the Embden-Meyerhof-Parnas (EMP) pathway yields 2 ATP per glucose whereas the Entner-Doudoroff (ED) pathway in *Z. mobilis* yields 1 ATP [68]. Indeed, biomass yields of *Z. mobilis* in anaerobic cultures (0.03-0.04 g·g glucose^{-1}) are much lower than those of *S. cerevisiae* (0.09-0.10 g·g glucose^{-1}) and actively growing cultures of *Z. mobilis* exhibit a considerably higher ethanol yield (0.48-0.49 g·g^{-1}) than *S. cerevisiae* (around 0.40 g·g^{-1}) [41, 46, 59]. The option to increase ethanol yields in *S. cerevisiae* by replacing its EMP glycolytic pathway by an ED pathway has been patented [45], but, to our knowledge, not further explored.

For some engineered pathways, the net ATP yield equals zero. An example is lactate production by engineered homolactic *S. cerevisiae* strains [78]. In contrast to lactic acid bacteria, which use lactate production as their primary catabolic pathway, an ATP requirement for export of lactic acid in *S. cerevisiae* cancels out the ATP produced in the EMP pathway [78]. Stable, robust product formation via 'zero-ATP pathways' requires the simultaneous activity of other ATP forming reactions to meet free-energy demands for growth and maintenance. In the case of lactic acid producing *S. cerevisiae* strains, this can be achieved through aerobic respiration [2, 78]. However, aeration of industrial-scale fermentations is expensive and results in a partial conversion of sugar to CO_2 at the expense of product formation. Alternatively, alcoholic fermentation can provide the required ATP [4]. This twin-product scenario, however, requires additional downstream processing and causes a dependency on two different markets.

The ideal scenario for production of commodity chemicals and biofuels involves fermentative, anaerobic pathways with a low, but positive ATP yield. Only such pathways enable low-cost anaerobic processes with a minimal production of excess biomass. Lactate production by lactic acid bacteria and (bio)ethanol production by *S. cerevisiae* are classical examples of such processes. In addition, several processes that are the result of metabolic engineering fit this description. For instance, the pathway from glucose to isobutanol by engineered *Escherichia coli* strains [5] is a typical fermentation pathway with an ATP yield of 2 mol·mol glucose^{-1}. In addition to enabling high product yields, such fermentative pathways exhibit a strict coupling of growth and product formation, which simplifies the use of evolutionary engineering to select for

improved productivity. This possibility has been extensively exploited for improvement of engineered *S. cerevisiae* strains that express heterologous pathways for pentose fermentation [10, 42, 43, 66, 87, 88].

Its impact on process economy makes the ATP stoichiometry of product pathways and precursor supply a highly relevant target in the design of metabolic engineering strategies. This mini-review does not aim to provide a full, in-depth analysis of possible strategies. Instead, it discusses possibilities and challenges involved in modifying energy coupling in product pathways by discussing selected examples in *S. cerevisiae*. This discussion will be focused on four selected reaction steps that occur in many industrially relevant product pathways that use glucose or other sugars as carbon and energy source: (i) uptake and phosphorylation of sugars, (ii) energy coupling of aerobic respiration and membrane-bound H^+-ATPases, (iii) $C_3 \rightarrow C_4$ carboxylation reactions and (iv) product export under industrial conditions. The final section provides a brief outlook.

Uptake and phosphorylation of sugars

Uptake, phosphorylation and, in the case of oligosaccharides, cleavage to yield monosaccharides are, not necessarily in this order, among the initial steps in microbial sugar metabolism. As will be discussed below, these reactions provide attractive opportunities to modulate the efficiency of free-energy (ATP) conservation by metabolic engineering.

In *S. cerevisiae*, the monosaccharides glucose, fructose and galactose are imported via facilitated diffusion (Figure 1.1), a process that does not require input of free energy and is mediated by members of the *HXT* (hexose transporter) family [11, 44]. Anaerobic fermentation of glucose to ethanol via the EMP pathway yields 2 ATP. In contrast, maltose transport in *S. cerevisiae* occurs via symport with a proton [75]. At typical values of the proton-motive force (PMF) of –150 to –200 mV, the free energy available from proton translocation across the yeast plasma membrane is –15 to –19 $kJ \cdot (mol\ H^+)^{-1}$ [57, 65]. Symport of maltose with a proton therefore enables transport against concentration gradients of up to 1000-fold. To maintain PMF and intracellular pH, proton import via the maltose symporter is balanced by proton export via the plasma-membrane H^+-ATPase. In *S. cerevisiae*, this requires the hydrolysis of 1 ATP for export of a single proton [75, 84]. Once inside the yeast cell, maltose

is hydrolyzed by maltase and the resulting two glucose molecules are converted via the EMP pathway. The net requirement of 1 ATP for maltose uptake limits the ATP yield of maltose fermentation to 3 ATP, which is equivalent to 1.5 ATP per glucose. Consistent with this lower ATP yield, the anaerobic biomass yield of *S. cerevisiae* on maltose is 25% lower than the biomass yield on glucose and, because more maltose is fermented to ethanol to energize maltose-proton symport, the ethanol yield on maltose is 9% higher than on glucose [84].

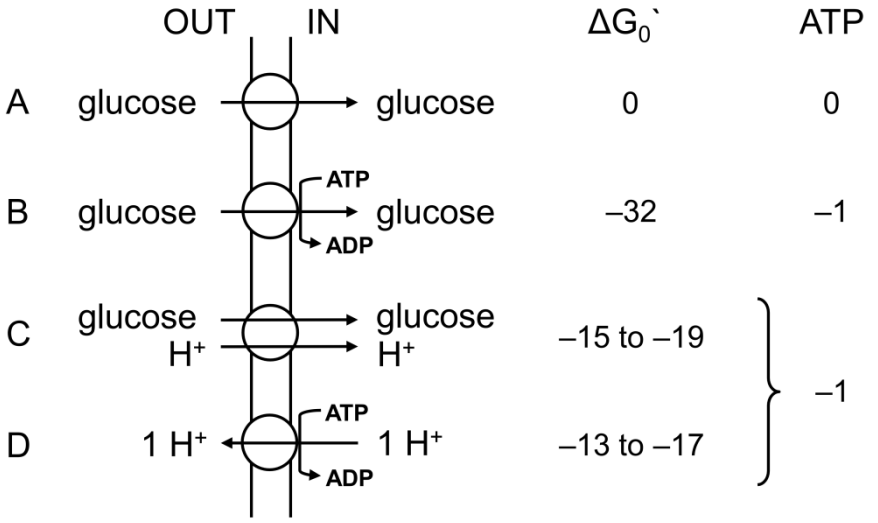

Figure 1.1 Different mechanisms for glucose import, for which the standard Gibbs free-energy change (ΔG_0`, in kJ·mol^{-1}) and net ATP requirement for import are indicated. Mechanisms: A: facilitated diffusion, B: active transport via an ABC-transporter, C: proton symport, D: plasma-membrane H^+-ATPase.

A recent metabolic engineering study [7] explored the possibility to use sugar-proton symport to increase the ethanol yield of *S. cerevisiae* on sucrose, the major sugar substrate for bioethanol production from cane sugar. Sucrose hydrolysis by wild-type *S. cerevisiae* is predominantly catalyzed by extracellular invertase [7, 8, 13, 15, 24, 72]. When the subsequent uptake of the hydrolysis products, glucose and fructose, by *HXT* transporters is followed by alcoholic fermentation via the EMP pathway, this yields 4 ATP per sucrose. *S. cerevisiae* also harbours a low capacity sucrose-proton-symport mechanism and, in wild-type strains, a low fraction of the invertase is present intracellularly [8, 15, 24,

61, 69]. Complete rerouting of sucrose metabolism via proton symport and intracellular hydrolysis was achieved by a combination of metabolic and evolutionary engineering. This resulted in a 30% decrease of the biomass yield and an 11% increase of the ethanol yield relative to a reference strain [7].

In principle, replacement of facilitated diffusion systems by proton symporters or, alternatively, by ATP-driven transporters (Figure 1.1) can be applied to other industrially relevant sugars, products and microorganisms to improve product yields of pathways with 'excess' ATP production. When applied to monosaccharides, the anticipated impact of this approach is even higher than for disaccharides. Theoretically, replacement of the *HXT* transporters in *S. cerevisiae* by a hexose-proton symport mechanism should decrease the biomass yield of anaerobic yeast cultures on hexose sugars by 50%, with a concomitant increase of the ethanol yield of 14% [7]. Interestingly, in literature such proton-symporters have been described for fructose (in *S. pastorianus* and wine yeasts [25]), glucose, arabinose and xylose (in several non-*Saccharomyces* yeasts [38]). Combined with the availability of strains in which all 20 genes of the *HXT* transporter family have been deleted [86], this presents an opportunity to explore the energetic impact of the introduction of such heterologous transporters in *S. cerevisiae*.

The initial intracellular reactions in *S. cerevisiae* sugar metabolism provide interesting opportunities to improve energy coupling in product pathways with a negative or zero ATP yield (Figure 1.2). Hexose kinases conserve only a fraction of the free energy that is available from the cleavage of ATP to ADP (ΔG_0` –32 kJ·mol$^{-1}$, but the *in vivo* $\Delta G$ can be estimated at –45 kJ·mol$^{-1}$ from previously published data [14]) in the resulting glucose-phosphate bond ($\Delta G_0$` –14 kJ·mol^{-1} for glucose-6-phosphate hydrolysis). Similarly, disaccharide hydrolases dissipate the free energy of disaccharide cleavage (ΔG_0` –16 kJ·mol^{-1} for maltose hydrolysis by maltase). In contrast, the phosphorolytic cleavage of disaccharides with inorganic phosphate, as catalyzed by disaccharide phosphorylases, is coupled to phosphorylation of one of the monosaccharides and thereby converses free energy. For example, the maltose phosphorylase (ΔG_0` of +5 kJ·mol$^{-1}$) reaction yields glucose and glucose-1-phosphate (whose formation from glucose and inorganic phosphate has a $\Delta G_0$` of +21 kJ·mol^{-1}) (Figure 1.2). Glucose-1-phosphate can subsequently be converted with a phosphoglucomutase into glucose-6-phosphate, the first intermediate of glycolysis. Although maltose phosphorolysis, which does not naturally occur in

S. cerevisiae, has a slightly positive ΔG_0` (+5 kJ·mol^{-1}, Figure 1.2), the actual ratio between the substrate and product concentrations creates a negative actual Gibbs free-energy change, thereby allowing *in vivo* functioning of this reaction in several microorganisms [23, 71].

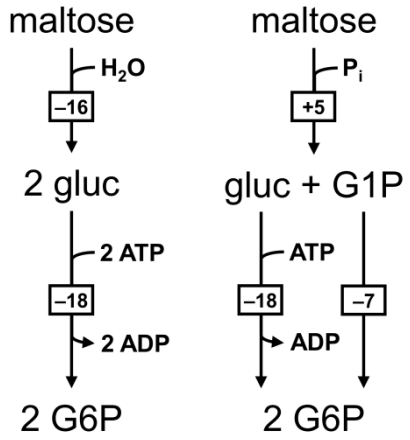

Figure 1.2 The ATP yield on maltose can be increased by replacing maltose hydrolysis (ΔG_0` –16 kJ·mol$^{-1}$) with maltose phosphorolysis ($\Delta G_0$` +5 kJ·mol^{-1}). Maltose phosphorylase cleaves maltose with inorganic phosphate and directly yields glucose-1-phosphate, which saves the hydrolysis of 1 ATP molecule during glucose phosphorylation by hexokinase. Standard Gibbs free-energy changes (ΔG_0`, in kJ·mol^{-1}) are indicated in boxes. Abbreviations: gluc: glucose, G1P: glucose-1-phosphate, G6P: glucose-6-phosphate.

Replacement of a disaccharide hydrolysis by a disaccharide phosphorolysis via metabolic engineering should, in theory, lead to a gain of one ATP per disaccharide molecule. This hypothesis was recently tested by replacing the native *S. cerevisiae* maltase by a maltose phosphorylase. Indeed, deletion of all maltase-encoding genes and introduction of a heterologous maltose phosphorylase gene in *S. cerevisiae* resulted in a 26% increase of the anaerobic biomass yield on maltose [20]. This concept should, in principle, be applicable to other disaccharides, such as cellobiose [60], sucrose [22] and lactose [18]. However, the ATP yield of the engineered *S. cerevisiae* strain expressing maltose phosphorylase (2 ATP·hexose molecule^{-1}) is still only equivalent to that of glucose metabolism (2 ATP) or of extracellular maltose hydrolysis followed by

monosaccharide uptake via facilitated diffusion (a mechanism that occurs, for example, in *Schizosaccharomyces pombe* [32]).

To enable conversion of 'zero-ATP' pathways such as malic and lactic acid production by engineered *S. cerevisiae* strains [2, 78, 93, 94] into pathways with a positive ATP yield, engineering of disaccharide metabolism needs to be taken one step further. One approach towards achieving this goal is to combine maltose phosphorylase expression with the replacement of the maltose transporter with a maltose facilitator, thereby eliminating the energy costs for maltose uptake. This strategy should yield 5 ATP for the anaerobic fermentation of maltose, equivalent to 2.5 ATP per hexose. To date, no disaccharide facilitators have been functionally expressed in *S. cerevisiae*. In *E. coli*, however, single-amino-acid changes have been shown to convert the lactose-proton symporter *lacY* of *E. coli* into a lactose facilitator [26]. When combined with the expression of a heterologous lactose phosphorylase [18], these *lacY* alleles may be applicable for proof-of-principle experiments in *E. coli*.

A strategy to even further increase ATP yields on hexose equivalents is suggested by an elegant study on metabolism of cellodextrins in *Clostridium thermocellum* [97]. In this anaerobic bacterium, cellodextrin oligosaccharides of up to 6 glucose units are all transported via the same ATP-driven transport mechanism, followed by their intracellular phosphorolytic cleavage. As a result, the relative impact of the ATP cost for transport decreases and the ATP yield per hexose equivalent increases with increasing chain length [97]. Phosphorolytic cleavage of oligosaccharides with more than 2 hexose units thereby offers the potential to increase ATP yields per hexose unit above 2.5. For example, a scenario in which the trisaccharide maltotriose is taken up by facilitated diffusion and cleaved by a heterologous maltotriose phosphorylase yields 8 ATP per maltotriose, or 2.67 ATP per hexose unit [20]. Obviously, any industrial implementation of such strategies is dependent on cost-effective strategies to produce feedstocks with a high content of the relevant oligosaccharides.

Stoichiometries of oxidative phosphorylation and H^+-ATPase

When redox constraints or ATP requirements preclude the use of anaerobic process conditions, aerobic respiration provides a means to reoxidize reduced cofactors and yield additional ATP. Aerobic respiration of redox cofactors is a

highly exergonic process (ΔG_0` –220 kJ·mol^{-1} for NADH and –200 kJ·mol^{-1} for $FADH_2$). During respiration, free energy is conserved as a proton-motive force across the mitochondrial inner membrane. This proton-motive force can subsequently be used to energize ATP production via the mitochondrial proton-translocating ATP synthetase complex. The H^+/ATP ratio of ATP synthetases in respiring membranes of microorganisms is consistently found to be close to 3 [83]. Conversely, the number of protons translocated during respiration of, for example, NADH strongly varies between microorganisms and cultivation conditions, depending on the composition of the respiratory chain and the proton coupling of individual components. The combined efficiency of proton pumping by respiratory chains and ATP synthesis is represented by the P/O ratio (ATP formed per electron pair transferred to oxygen) [36].

When respiration of reduced cofactors yields more ATP than is required in the product pathway, this may contribute to the formation of excess biomass and reduce the rate of product formation via respiratory coupling [16]. In such cases, decreasing the P/O ratio provides an attractive strategy to increase product yields. Two metabolic engineering strategies have been described that reduce the P/O ratio of respiration in *S. cerevisiae*. The first strategy is based on the occurrence, in many fungi, of alternative oxidases that transfer electrons from cytochrome c to molecular oxygen without proton translocation [35]. Indeed, expression of alternative oxidases in *S. cerevisiae* led to a small but significant reduction of the biomass yield in aerobic cultures [30, 31, 80]. A second strategy that completely bypasses the respiratory chain, is the expression of a bacterial water-forming NADH oxidase in *S. cerevisiae*. Since this system does not donate electrons from NADH to the respiratory chain, but directly to oxygen, its expression results in a decreased aerobic biomass yield [29, 30, 80]. Expression of a soluble NADH oxidase only had a small impact on biomass yields in glucose-limited aerobic chemostat cultures (ca. 10% reduction [80]). This can be explained from its targeting to the cytosol, due to which the P/O ratio for respiration of intramitochondrial NADH, which makes up the majority of the NADH formed during respiratory metabolism of glucose, is likely to have been unaffected.

For product pathways that do not yield ATP, the ATP yield and P/O ratio should be maximized to minimize the amount of carbon substrate that needs to be respired to generate ATP for growth and cellular maintenance. In contrast to many other yeasts and fungi, the mitochondrial respiratory chain of

S. cerevisiae does not contain a proton-translocating complex I-type NADH dehydrogenase. Instead, *S. cerevisiae* uses a single-subunit, non-proton-translocating NADH dehydrogenase (Ndi1p) to couple oxidation of intramitochondrial NADH to the respiratory chain [6]. As a result, its effective P/O ratio is low (close to 1) and complete glucose oxidation in *S. cerevisiae* probably only yields 16 ATP [6, 74]. Since eukaryotic complex I-type NADH dehydrogenases consist of 40-45 different protein subunits [37], a strategy to functionally express, assemble and integrate such a complex in the mitochondrial inner membrane of *S. cerevisiae* represents a formidable metabolic engineering challenge.

With a proton-ATP stoichiometry close to 3 [83], the H^+-ATP synthetases in respiring microbial membranes are thermodynamically very efficient (at a proton-motive force of –150 mV, inward translocation of a single proton represents a free-energy change of –15 $kJ \cdot mol^{-1}$, while the *in vivo* ΔG for ATP hydrolysis in *S. cerevisiae* is around –45 $kJ \cdot mol^{-1}$ [14]). A completely different situation exists for the plasma-membrane H^+-ATPase in *S. cerevisiae*, which couples the hydrolysis of an ATP molecule to the translocation of only a single proton and therefore appears to operate far from maximum thermodynamic efficiency [75, 84]. Increasing the stoichiometry of the yeast plasma-membrane H^+-ATPase from 1 to 2 H^+/ATP could decrease the amount of ATP consumed for maintenance processes and increase the ATP yield of processes that involve proton-coupled transport of substrates (e.g. maltose and NH_4^+) [48, 75, 84] or products (e.g. organic acids) [3, 62, 77]. For example, alcoholic fermentation of a disaccharide, using a disaccharide-proton symporter and disaccharide phosphorylase, combined with an H^+-ATPase stoichiometry of 2 H^+/ATP, would yield 4.5 ATP per maltose (2.25 ATP per hexose). Moreover, an increased H^+-ATPase stoichiometry might improve tolerance to both low pH and weak organic acids [1, 54, 81]. Altering the H^+/ATP stoichiometry of the plasma-membrane H^+-ATPase of *S. cerevisiae* therefore presents a promising metabolic engineering strategy. Interestingly, specific single-amino-acid changes in the *S. cerevisiae* plasma-membrane H^+-ATPase Pma1p have been reported that led to increased *in vitro* H^+/ATP stoichiometries [27, 53]. However, these 'hypercoupled' isoforms did not show increased *in vivo* stoichiometries [19]. Further studies into the proton coupling of eukaryotic plasma-membrane H^+-ATPases therefore remain of great fundamental and applied interest.

$C_3 \rightarrow C_4$ carboxylation reactions

Carboxylation reactions couple inorganic carbon (CO_2 or bicarbonate) to organic acceptor molecules. Examples of industrially relevant processes that involve carboxylation steps include the production of C_4-dicarboxylic acids, lipids, polyketides, flavonoids, propionic acid and 3-hydroxypropionic acid [28, 34, 77]. In some of these processes, such as for example lipid synthesis, carboxylation occurs as an intermediate step and is followed by a loss of CO_2. In other cases, such as production of C_4-dicarboxylic acids, a net fixation of CO_2 can occur.

Production of malic and succinic acid is a key topic in metabolic engineering and strategies for high-yield production of these compounds from sugars depend on reactions that carboxylate the glycolytic C_3-intermediates pyruvate or phosphoenolpyruvate (PEP) [3, 9, 56, 67, 89, 90]. Energy coupling of these carboxylation reactions has a strong impact on the maximum yields of C_4-dicarboxylic acid production.

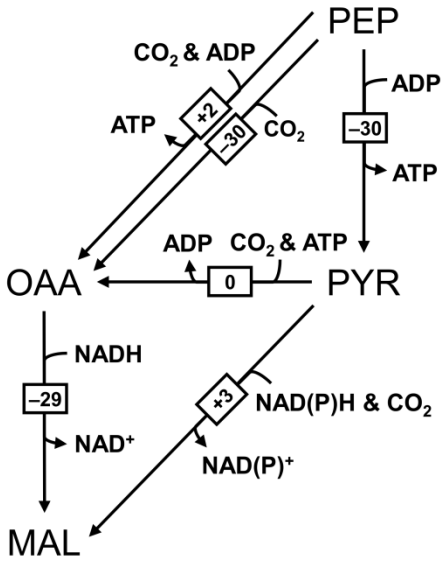

Figure 1.3 Carboxylation of glycolytic C_3-intermediates to C_4-dicarboxylic acids (e.g. malic acid) can be mediated via several mechanisms with different efficiencies of free-energy (ATP) conservation. Standard Gibbs free-energy changes (ΔG_0`, in $kJ \cdot mol^{-1}$) are indicated in boxes. Abbreviations: PEP: phosphoenolpyruvate, PYR: pyruvate, OAA: oxaloacetate, MAL: malic acid.

Carboxylation of pyruvate to oxaloacetate with CO_2 requires the input of free energy (ΔG_0` +32 kJ·mol$^{-1}$). In the reaction catalyzed by pyruvate carboxylase, this free energy is provided by ATP hydrolysis, leading to a $\Delta G_0$` of 0 kJ·mol^{-1}. Subsequent reduction of oxaloacetate yields a redox-cofactor balanced, CO_2-fixing pathway from glucose to two molecules of malic acid. However, the zero ATP yield of this pathway precludes efficient production under anaerobic conditions, since another pathway – usually aerobic respiration – is required to supply the cells with ATP for growth and cellular maintenance [91, 92]. Similarly, redox-neutral pathways for production of succinic acid via pyruvate carboxylase and either the glyoxylate shunt or the oxidative branch of the TCA-cycle only yield 0.33 ATP per succinic acid [33, 79]. Since export of C_4-dicarboxylic acids under industrially relevant conditions (high product titers and preferably a low pH to facilitate product recovery [3]) is likely to require an additional input of free energy (e.g. 1 ATP per C_4-acid, see next section) [3, 77], increasing the ATP yield of product formation is a key objective for metabolic engineering of the production of C_4-dicarboxylic acids.

Several opportunities are available to increase free-energy (ATP) conservation of $C_3 \rightarrow C_4$ carboxylation reactions in C_4-dicarboxylic acid production (Figure 1.3). For instance, malic enzyme (pyruvate + CO_2 + NAD(P)H → malic acid + NAD(P)$^+$, ΔG_0` +3 kJ·mol$^{-1}$) combines the free energy from the reduction of oxaloacetate to malic acid ($\Delta G_0$` –29 kJ·mol^{-1}) to the carboxylation of pyruvate (ΔG_0` +32 kJ·mol$^{-1}$). Alternatively, the energy-rich intermediate PEP ($\Delta G_0$` –62 kJ·mol^{-1} upon hydrolysis) can be used as substrate instead of pyruvate. Direct carboxylation of PEP to oxaloacetate by PEP carboxylase (ΔG_0` –30 kJ·mol$^{-1}$) has been investigated for succinic acid production by engineered *E. coli* strains [49], but does not yield additional ATP compared to pyruvate carboxylase. The remaining free energy of the carboxylation of PEP to oxaloacetate ($\Delta G_0$` –30 kJ·mol^{-1}) can be conserved via phosphorylation of ADP to ATP, mediated by PEP carboxykinase (ΔG_0` +2 kJ·mol^{-1}). Therefore, using either malic enzyme or PEP carboxykinase instead of pyruvate carboxylase increases the overall ATP yield by 1 ATP per $C_3 \rightarrow C_4$ carboxylation event. These replacements in theory enable anaerobic malic acid production and drastically increase the (marginal) ATP yield for redox-neutral succinic acid production from 0.33 to 1.17 ATP (together with the glyoxylate shunt) or 1.33 ATP (together with the oxidative TCA-cycle) per succinic acid [39, 70, 93-96].

Product export under industrial conditions

Product export across the plasma membrane is the final step in most yeast-based fermentation processes. Product export can be mediated via several mechanisms, whose different energy coupling strongly affects both the maximum achievable concentration gradients across the membrane and net ATP formation (for an overview, see [77]). The impact of the export mechanism on product formation is illustrated by succinic acid production under industrial conditions (i.e. high titer and low pH) with *S. cerevisiae*. Succinic acid is a dicarboxylic acid (pK_a's: 4.21 and 5.64) and therefore, depending on the pH, exists as undissociated acid (H_2Suc), monovalent anion ($HSuc^-$) and divalent anion (Suc^{2-}). To minimize the addition of base during fermentation and acid during downstream processing, respectively, preferably the undissociated acid (H_2Suc) is produced at low extracellular pH. However, at the near-neutral pH values inside the cell, weak organic acids predominantly exist as divalent anions (Suc^{2-}), which has major consequences on the export energetics. Thermodynamic analysis of dicarboxylic acid production [3, 77] indicated that facilitated diffusion of the undissociated acid and the monovalent anion (or the divalent anion together with one proton) do not support the concentration gradients required for high titer (e.g. 1 M) succinic acid production at low pH (e.g. pH 3). In addition, facilitated diffusion of the divalent anion was only found thermodynamically feasible at relatively high intracellular succinic acid concentrations (around 0.1 M) and even then would still require the subsequent extrusion of 2 protons by the plasma-membrane H^+-ATPase. An additional thermodynamic push through antiport of the divalent anion (Suc^{2-}) with one proton (or thermodynamically equivalent reaction) would allow export at low intracellular concentrations (<0.01M), but requires the simultaneous expulsion of 3 protons. Since the plasma-membrane H^+-ATPase in *S. cerevisiae* has a stoichiometry of one, this would respectively costs 2 and 3 ATP per succinic acid exported, unless the H^+/ATP stoichiometry of the plasma-membrane H^+-ATPase can be increased as described above whilst maintaining the same proton-motive force [19]. Alternatively, it might be possible to directly couple succinic acid (H_2Suc) export to ATP hydrolysis via primary transport, thereby reducing the export costs to only 1 ATP. This illustrates that succinic acid export under industrially desirable conditions (high titer and low pH) thus

always requires the investment of free energy (ATP) and thereby influences the overall ATP yield of succinic acid production from glucose.

Discussion and outlook

The examples discussed above illustrate how targeted modification of energy coupling of key reactions in product pathways can drive product yields of engineered yeast strains closer to their thermodynamic and stoichiometric maximum. Combined modification of multiple of these reactions may enable ATP-positive metabolic pathways to products of interest and thereby facilitate evolutionary engineering of strains through the coupling between product formation and growth [63]. In addition to their impact on product yield, such engineering strategies may also have a profound impact on process design when they enable a switch from aerobic to anaerobic process conditions, thereby eliminating aeration and associated costs of stirring, air compression and cooling.

Many other targets for metabolic engineering of energy coupling exist in addition to the ones discussed in this mini-review. One extensively studied strategy that has not been discussed is the introduction of ATP-hydrolyzing (sets of) reactions. Such futile cycles provide a powerful alternative means of modulating ATP stoichiometries [17, 51]. However, optimization of product yields and cellular robustness requires a careful balancing of ATP synthesizing and ATP hydrolyzing reactions which, in the case of futile cycles, may be difficult to maintain under dynamic industrial conditions. Another important metabolic node at which optimization of energy coupling can have a strong impact on product yields is the formation of Coenzyme A (CoA) esters. This is illustrated by the energy coupling of the synthesis of acetyl-CoA, a key precursor for a wide range of biotechnological products. In *S. cerevisiae*, formation of cytosolic acetyl-CoA via cytosolic acetyl-CoA synthetases costs 2 ATP equivalents. In contrast, synthesis of this precursor via the mitochondrial pyruvate dehydrogenase does not require a net input of ATP, thus illustrating the importance of metabolic compartmentation in the optimization of product pathways in yeast [55].

Microbial metabolic diversity provides an endless source of inspiration for novel metabolic engineering strategies to improve free-energy (ATP) conservation in industrial microorganisms such as *S. cerevisiae*. An elegant and

efficient example is provided by propionic acid bacteria [64]. In a single pathway, these microorganisms combine three evolutionary 'innovations' to achieve a near-perfect conservation of free energy: (i) ATP-independent integration of a $C_3 \rightarrow C_4$ carboxylation with a $C_4 \rightarrow C_3$ decarboxylation, (ii) an ATP-independent CoA-transfer reaction that circumvents ATP-dependent formation of a CoA-ester and (iii) ATP formation from fumarate respiration, which couples the reduction of fumarate with NADH to proton translocation and subsequent ATP synthesis [21, 40].

Systematic exploration of metabolic biodiversity for the design of energy-efficient pathways can be intensified by mathematical models that enable a combinatorial, parallel evaluation of possible pathway configurations, by analyzing ATP yield as well as pathway thermodynamics [28, 34]. Such a model-based analysis, which also includes pathways based on predicted, but currently non-confirmed, enzyme activities, has been successfully performed for 3-hydroxypropionic acid production [28, 34]. Further functional analysis and exploration of hitherto undiscovered metabolic processes remains essential, since model prediction are only as good as the questions asked and the possibilities used as inputs [82].

As pathways are redesigned to increase free-energy (ATP) conservation, the overall Gibbs free-energy change of the reaction by definition becomes less negative. In some cases, increased ratios of substrate and product concentrations or reactions elsewhere in the pathways can (partially) compensate for this decrease of the driving force for product formation. For example, replacing maltose hydrolysis (ΔG_0` –16 kJ·mol$^{-1}$) with maltose phosphorolysis ($\Delta G_0$` +5 kJ·mol^{-1}) in *S. cerevisiae* coincided with an almost three-fold higher residual maltose concentration in maltose-limited chemostat cultures [20]. Similarly, when the native ATP-consuming pyruvate carboxylase in *S. cerevisiae* was functionally replaced by the energetically more efficient malic enzyme or phosphoenolpyruvate carboxykinase reactions, readjustment of the intracellular metabolite pools, switching of the cofactor specificity from NADH to NADPH or even cultivating with increased CO_2 concentrations could only partially overcome the decreased driving forces, as illustrated by the strongly decreased growth rates of these strains [93, 94].

Strategies for improving free-energy coupling should additionally take into account the ATP demands for performance and robustness of the resulting strains under industrial conditions, such as high product concentrations, low

pH, weak organic acids and other inhibitory compounds [52]. Trade-offs between stoichiometry, kinetics and robustness should therefore be taken into account in the economic evaluations that ultimately decide whether modifications in free-energy (ATP) conservation are beneficial to process development.

Scope and outline of this thesis

Microbial production of fuels and chemicals provides an alternative to petrochemical production. To compete with oil-based products, not only the kinetics of product formation, but also the product yield needs to be optimized. Whereas reduction of byproduct formation, modification of redox-cofactor balances and optimization of the stoichiometry of product formation are common practices to improve the product yield, the ATP yield of a product pathway is often overlooked. As illustrated in **chapter 1**, the ATP yield of product formation is however of paramount importance for the product yield that can be obtained. Several opportunities for improvement of free-energy (ATP) conservation in the yeast *Saccharomyces cerevisiae*, a key industrial microorganism, through metabolic engineering are presented.

An illustrative example of the importance of increased free-energy (ATP) conservation is lactic acid production with engineered *S. cerevisiae* strains. In terms of NAD(H) and ATP yield, conversion of glucose to lactic acid is equivalent to ethanol production. Whereas ethanol can be produced efficiently under anaerobic conditions, homolactic *S. cerevisiae* strains cannot grow under anaerobic conditions, presumably because export of 1 molecule lactic acid requires the net hydrolysis of 1 molecule ATP. This results in a 'zero-ATP pathway' and thereby precludes efficient production under anaerobic conditions. The goal of this PhD project was therefore to study concepts for the improvement of free-energy (ATP) conservation that potentially can enable anaerobic lactic acid production by *S. cerevisiae*.

Hitherto, the identity of the gene(s) responsible for lactic acid export in *S. cerevisiae* and the energy-coupling mechanism(s) of the encoded transporter(s) are unknown. Jen1p is the only *S. cerevisiae* lactate transporter reported in literature and is required for import of and growth on lactic acid. To identify alternative lactate transporters, a *jen1*Δ strain was evolved for growth on lactic acid (**chapter 2**). The evolved strains were subjected to a combination of transcriptome analysis and whole-genome DNA (re)sequencing to identify the relevant mutations. The resulting lead genes were tested for their lactate transport activity via knockout studies in the evolved strains and introduction into non-evolved strains.

Alternative strategies to obtain extra ATP from the conversion of substrate into product are highly relevant for anaerobic production of lactic acid

and other 'zero-ATP products' by *S. cerevisiae*. In **chapter 3**, the hydrolysis of maltose was replaced by maltose phosphorolysis to increase free-energy (ATP) conservation during alcoholic fermentation of this disaccharide. In theory, direct formation of glucose-1-phosphate by maltose phosphorylase saves the hydrolysis of one ATP per maltose in the hexokinase reaction to phosphorylate glucose to glucose-6-phosphate and increases the ATP yield from 3 to 4 ATP per maltose. To analyze the quantitative impact of such a replacement on free-energy (ATP) conservation in anaerobic maltose-grown cultures, the native maltose metabolism of *S. cerevisiae* was deleted and a bacterial maltose phosphorylase was introduced. Subsequently, enzyme activities, growth rates and biomass yields on maltose were analyzed in batch and chemostat cultures.

Another possible strategy for improving free-energy (ATP) conservation is based on the introduction of a plasma-membrane H^+-ATPase with an increased H^+/ATP stoichiometry. The H^+/ATP stoichiometry of the plasma-membrane H^+-ATPase determines the ATP requirement for cellular homeostasis and maintenance of the proton-motive force. In this way the H^+/ATP stoichiometry influences the biomass yield on substrates whose import makes use of the proton-motive force (e.g. maltose and NH_4^+), influences tolerance to both low pH and weak organic acids and can have a crucial impact on the stoichiometry and kinetics of organic acid production by engineered strains of *S. cerevisiae*. In **chapter 4**, the *in vivo* proton stoichiometry of two isoforms (Ser800Ala and Glu803Gln) of the plasma-membrane H^+-ATPase Pma1p, which previously showed increased *in vitro* H^+/ATP stoichiometries, was investigated. To this end, the corresponding mutant alleles were introduced in *S. cerevisiae* strains and the anaerobic biomass yields on maltose, which is imported via a proton-symport mechanism, were measured.

'Zero-ATP processes' such as lactic and malic acid production by engineered *S. cerevisiae* strains could benefit from the strategies for increased free-energy (ATP) conservation that were investigated in chapters 3 and 4. Conversely, industrial processes such as bio-ethanol production, in which product formation is coupled to a net production of ATP, might benefit from decreased free-energy conservation, since ATP enables formation of excess biomass and thereby decreases the product yield. The (industrial) fermentation of sucrose to ethanol by *S. cerevisiae* proceeds predominantly via extracellular hydrolysis of the disaccharide sucrose into glucose and fructose. Subsequent uptake of these monomeric sugars via facilitated diffusion and conversion to

ethanol yields 4 ATP. Alternatively, sucrose can be imported via a sucrose-proton-symport mechanism and hydrolyzed inside the cell, which only yields 3 ATP due to the ATP cost of sucrose import. As a result, intracellular sucrose hydrolysis theoretically yields 25% less biomass and 9% more ethanol than extracellular hydrolysis. The goal of **chapter 5** was to investigate whether relocation of sucrose hydrolysis from the extracellular space to the cytosol can be used to improve ethanol yields on sucrose and which additional steps may be required to improve sucrose utilization by strains that only express intracellular invertase.

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Chapter 2

Laboratory evolution of new lactate transporter genes in a *jen1*Δ mutant of *Saccharomyces cerevisiae* and their identification as *ADY2* alleles by whole-genome resequencing and transcriptome analysis

Stefan de Kok, Jurgen F. Nijkamp, Bart Oud, Filipa C. Roque, Dick de Ridder, Jean-Marc Daran, Jack T. Pronk and Antonius J.A. van Maris

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Abstract

Laboratory evolution is a powerful approach in applied and fundamental yeast research, but complete elucidation of the molecular basis of evolved phenotypes remains a challenge. In this study, DNA microarray-based transcriptome analysis and whole-genome resequencing were used to investigate evolution of novel lactate transporters in *Saccharomyces cerevisiae* that can replace Jen1p, the only documented *S. cerevisiae* lactate transporter. To this end, a *jen1*Δ mutant was evolved for growth on lactate in serial batch cultures. Two independent evolution experiments yielded Jen1p-independent growth on lactate as sole carbon source (μ_{max} 0.14 and 0.18 h^{-1} for single-cell lines IMW004 and IMW005, respectively). Transcriptome analysis of two independently evolved strains did not provide leads but whole-genome resequencing showed different single-nucleotide changes (C755G/Leu219Val and C655G/Ala252Gly) in the acetate transporter gene *ADY2*. Introduction of these *ADY2* alleles in a *jen1*Δ *ady2*Δ strain enabled growth on lactate (μ_{max} 0.14 h^{-1} for Ady2p^{Leu219Val} and 0.12 h^{-1} for Ady2p^{Ala252Gly}), demonstrating that these two alleles of *ADY2* (C655G and C755G) encode efficient lactate transporters. Analysis of mRNA expression levels and depth of coverage of DNA sequencing, combined with karyotyping, gene deletions and diagnostic PCR showed that, in IMW004, an isochromosome III (ca. 475 kb) with two additional copies of *ADY2*^{C755G} had been formed via crossover between retrotransposons *YCLWΔ15* and *YCRCΔ6*. The formation of this isochromosome shows how even a short period of selective pressure can cause substantial karyotype changes.

Introduction

Laboratory evolution (referred to as 'evolutionary engineering' in applied contexts) is a powerful strategy to obtain microorganisms with improved characteristics that are difficult to engineer through rational approaches, especially when the genetic determinants for a phenotype are unknown or complex [47]. Subsequent discovery of the underlying mutation(s) is of key importance, both for functional gene analysis and to enable transfer of the acquired traits to non-evolved industrially relevant strain backgrounds ('reverse metabolic engineering' [6]). In addition to the mutation(s) of interest, random mutations can accumulate during evolution. Combined with our incomplete understanding of metabolic and regulatory networks, this makes elucidation of the genetic basis of acquired phenotypes the main challenge of reverse metabolic engineering. During evolution, different types of mutations can occur: (i) small local changes (substitutions, insertions, deletions and duplications) that result in either differential gene expression or changes in the amino acid sequence of the encoded protein and/or (ii) rearrangements of larger DNA fragments, potentially resulting in a different gene dosage or expression [47].

Several genome-wide techniques have been described in literature to analyze mutations that occur during laboratory evolution (for reviews, see [10, 28, 30]). Classical techniques include construction and analysis of plasmid-based genomic libraries and quantitative trait locus mapping via crossing. More recent developments have enabled whole-genome DNA sequencing, DNA hybridization to DNA microarrays, transcriptome analysis using DNA microarrays, proteome, metabolome and fluxome profiling [10, 28, 30]. Analysis with DNA microarrays was, until recently, the only affordable genome-wide method. Microarray-based transcriptome analysis only indirectly indicates changes on the DNA level. Several factors, such as complex regulatory networks, transcription factor binding, translation efficiency and stability of mRNA and proteins complicate the interpretation of the exact genetic basis of changes observed at mRNA and protein level. In contrast, comparative genome hybridization using DNA microarrays can identify genetic changes directly [17, 49, 65]. However, limitations in the maximum probe density imply that either (i) the resolution of these DNA microarrays is limited to several base pairs, necessitating sequencing of selected regions or (ii) multiple DNA microarrays are required to cover larger (eukaryotic) genomes. Furthermore, DNA

microarrays can only be used for a comprehensive analysis when the strains under investigation are congenic to the strain on whose genome sequence the microarray design was based. In contrast, whole-genome DNA sequencing, which due to recent price developments has become an interesting technique for analysis of evolved strains [41, 48], has the potential to directly reveal all mutations accumulated in an evolved strain. However, discriminating between random and relevant mutations remains a challenging task. Combining DNA sequencing with other genome-wide techniques that provide insight in the underlying physiology can help to elucidate the genetic basis of a phenotype acquired via evolution.

Transport of carboxylic acids plays a key role in weak organic acid stress [44] and in metabolic engineering strategies for organic acid production with *Saccharomyces cerevisiae* [36, 58]. However, the responsible membrane transporters are often poorly studied and encoded by multiple redundant genes [13]. The goal of the present study was to investigate whether laboratory evolution and subsequent elucidation of the underlying mutations can lead to the identification of alternative lactate transporters in *S. cerevisiae*. In *S. cerevisiae*, Jen1p was previously identified as the only efficient lactate importer by generation of a UV-mutant unable to grow on lactate and subsequent functional complementation with a genomic library [12]. The lactate uptake rate of the resulting *jen1* null mutant was close to the detection limit [12]. Interestingly, export of lactate in engineered lactate producing *S. cerevisiae* strains is unaffected by deletion of *JEN1* (our unpublished results). These observations suggest the presence of at least one alternative lactate transporter. To explore the presence of other lactate transporters in *S. cerevisiae*, a *jen1*Δ strain was constructed and evolved for growth on lactate as the sole carbon and energy source. The evolved strains were subjected to a combination of transcriptome analysis and whole-genome DNA (re)sequencing to identify the relevant mutations. The resulting lead genes were tested for their lactate transport activity via knockout studies in the evolved strains and introduction into non-evolved strains.

Materials and Methods

Strains and maintenance

The *S. cerevisiae* strains used and constructed in this study (Table 2.1) are all derived from CEN.PK113-7D [21, 54]. Stock cultures were grown at 30 °C in

shake flasks containing either 100 ml synthetic medium [60] with 20 $g \cdot l^{-1}$ glucose as carbon source or 100 ml complex medium containing 20 $g \cdot l^{-1}$ glucose, 10 $g \cdot l^{-1}$ Bacto yeast extract and 20 $g \cdot l^{-1}$ Bacto peptone. After overnight growth, 20% (v/v) glycerol was added and 1-ml aliquots were stored at –80 ˚C.

Table 2.1 *Saccharomyces cerevisiae* strains used in this study.

Strain	Relevant genotype	Source
CEN.PK113-7D	MATa *URA3 ADY2 JEN1*	P. Kötter, Frankfurt
IMK302	MATa *URA3 ADY2 jen1::loxP-KanMX4-loxP*	This study
IMW004	IMK302 evolved for growth on lactate	This study
IMW005	IMK302 evolved for growth on lactate	This study
IMW032	IMW004 *ady2::loxP-hphNT1-loxP*	This study
IMW040	IMW004 *ady2::loxP-hphNT1-loxP ady2::loxP-natNT2-loxP*	This study
IMW041	IMW004 *ady2::loxP-hphNT1-loxP ady2::loxP-natNT2-loxP ady2::loxP-ble-loxP*	This study
IMW033	IMW005 *ady2::loxP-hphNT1-loxP*	This study
IMK322	MATa *ura3::loxP-hphNT1-loxP ADY2 jen1::loxP-KanMX4-loxP*	This study
IMK338	MATa *ura3::loxP ADY2 jen1::loxP*	This study
IMK341	MATa *ura3::loxP ady2::loxP-hphNT1-loxP jen1::loxP*	This study
IMZ271	IMK341 pRS416 (*CEN6/ARS4*, *URA3*)	This study
IMZ272	IMK341 pUDC011 (*CEN6/ARS4*, *URA3*, P_{ADY2}-*ADY2*-T_{ADY2})	This study
IMZ273	IMK341 pUDC012 (*CEN6/ARS4*, *URA3*, P_{ADY2}-*ADY2*^{C655G}-T_{ADY2})	This study
IMZ276	IMK341 pUDC015 (*CEN6/ARS4*, *URA3*, P_{ADY2}-*ADY2*^{C755G}-T_{ADY2})	This study

Table 2.2 Plasmids used in this study.

Plasmid	Characteristic	Reference/source
pCR-BLUNT II-TOPO	Gateway entry plasmid	Invitrogen, USA
pRS416	Centromeric plasmid, *URA3*	[50]
pUG6	PCR template for *loxP-KanMX4-loxP* cassette	[31]
pUG66	PCR template for *loxP-ble-loxP* cassette	[31]
pSH47	Centromeric plasmid, *URA3*, P_{GAL1}-*Cre*-T_{CYC1}	[31]
pUG-*hphNT1*	PCR template for *loxP-hphNT1-loxP* cassette	[18]
pFA6a-*natNT2*	Plasmid with *natNT2* marker	[35]
pUG-*natNT2*	PCR template for *loxP-natNT2-loxP* cassette	This study
pUD151	Gateway entry clone, P_{ADY2}-*ADY2*-T_{ADY2}	This study
pUD152	Gateway entry clone, P_{ADY2}-*ADY2*^{C655G}-T_{ADY2}	This study
pUD152	Gateway entry clone, P_{ADY2}-*ADY2*^{C755G}-T_{ADY2}	This study
pUDC011	Centromeric plasmid, *URA3*, P_{ADY2}-*ADY2*-T_{ADY2}	This study
pUDC012	Centromeric plasmid, *URA3*, P_{ADY2}-*ADY2*^{C655G}-T_{ADY2}	This study
pUDC015	Centromeric plasmid, *URA3*, P_{ADY2}-*ADY2*^{C755G}-T_{ADY2}	This study

Table 2.3 Primers used in this study.

Primer name	Sequence (5'→3')
JEN1 KO Fw	TGCACATCATTGTTGAGAAATAGTTTTGGAAGTTGTCTAGTCCTTCTCCC CAGCTGAAGCTTCGTACGC
JEN1 KO Rv	GAAATGCAGTTACATAGAGAAGCGAACACGCCCTAGAGAGCAATGAAA AGGCATAGGCCACTAGTGGATCTG
JEN1 Ctrl Fw	GCGCGGCTTGAAACTATTTCTCC
JEN1 Ctrl Rv	GGCCCATTCAGTGCAAGAACC
ADY2 KO Fw A	CACAGATATAACTAAACAACCACAAAACAACTCATATACAAACAAATAAT CAGCTGAAGCTTCGTACGC
ADY2 KO Rv A	CAATAGTTCTCGTTATTAGTAGGTCGTGCTCTTAAAAGATTACCCTTTCA GCATAGGCCACTAGTGGATCTG
ADY2 KO Fw B	ATGTCTGACAAGGAACAAACGAGCGGAAACACAGATTTGGAGAATGCA CCCAGCTGAAGCTTCGTACGC
ADY2 KO Rv B	GATGGTAATGGGAATGGACGAGCCAGTACATATGAATTCTGCTTTGTA GCGCATAGGCCACTAGTGGATCTG
ADY2 KO Fw C	GATACTATAGTTCCCATGATAACGACGTTAATGGCGTTGCAGAAGATGA ACCAGCTGAAGCTTCGTACGC
ADY2 KO Rv C	CTGCATATGCGTTGTACCAAGCAATGAAAGCAACAACAACTCCCAGGAC ACGCATAGGCCACTAGTGGATCTG
ADY2 Ctrl Fw	GGGCGGCTATTTCGGTATTG
ADY2 Ctrl Rv	GCAACATCTTCTCCGCTAACC
URA3 KO Fw	TCCTAGTCCTGTTGCTGCCAAGCTATTTAATATCATGCACGAAAAGCAAA CAAACTTGTGGGGCACACCCAATTTAGACACAGCTGAAGCTTGTACGC
URA3 KO Rv	TTCCCAGCCTGCTTTTCTGTAACGTTCACCCTCTACCTTAGCATCCCTTCC CTTTGCAAATAGGCGCGTCAAGTACAAAGTTAGCATGCCACTAGTGGAT CTG
URA3 Ctrl Fw	GCTACTGCGCCAATTGATGAC
URA3 Ctrl Rv	CGAGATTCCCGGGTAATAACTG
KanMX4 Ctrl Fw	TCGTATGTGAATGCTGGTCG
KanMX4 Ctrl Rv	CGCACGTCAAGACTGTCAAG
hphNT1 Ctrl Fw	ACGCGGATTTCGGCTCCAAC
hphNT1 Ctrl Rv	AGACGTCGCGGTGAGTTCAG
natNT2 Ctrl Fw	CCACTCTTGACGACACGGCTTAC
natNT2 Ctrl Rv	GTAAGCCGTGTCGTCAAGAGTGG
ble Ctrl Fw	CGAATTGCTTGCAGGCATCTC
ble Ctrl Rv	AATCTCGTGATGGCAGGTTGG
ADY2 inside Fw	GCCTTTGGTGGTACCTTGAATC
ADY2 inside Rv	GCTCTTGTGACACCAAGTCTATTAGC
ADY2p Fw	CACCACCCGGGAGGATCCGATAGGCGTCGTATATAGTCTCTTC
ADY2t Rv	GCACTAGTTCTAGACGCATTTGGATCGTCCAG
P5	TGCACCAGCAAATGAGGCTTC
P6	ATAGTCAATGCCCGTGGTTC

Plasmid and strain construction

The plasmids and primers used in this study are listed in Table 2.2 and 2.3, respectively. Transformations of *S. cerevisiae* were carried out according to the LiAc/ssDNA method [27]. Gene deletions were performed using the loxP-marker-loxP/Cre recombinase system, using pUG6, pUG66, pUG-*natNT2* and pUG-*hphNT1* as templates for PCR amplification of the knockout cassettes [31]. Selection of knockout mutants was performed on agar plates containing 20 $g \cdot l^{-1}$ glucose, 10 $g \cdot l^{-1}$ Bacto yeast extract, 20 $g \cdot l^{-1}$ Bacto peptone and either 200 $mg \cdot l^{-1}$ G418, 10 $mg \cdot l^{-1}$ phleomycin, 100 $mg \cdot l^{-1}$ nourseothricin or 200 $mg \cdot l^{-1}$ hygromycin B. The *natNT2* marker was cloned from pFA6a-natNT2 [35] to pUG6 via the *Sac*I and *Bgl*II restriction sites, thereby replacing the *KanMX4* marker, resulting in pUG-*natNT2*. The *JEN1* knockout cassette was amplified from pUG6 using primers JEN1 KO Fw and JEN1 KO Rv and transformed to CEN.PK113-7D, resulting in strain IMK302. Correct integration of the knockout cassette was confirmed using primer combinations JEN1 Ctrl Fw/KanMX4 Ctrl Rv and KanMX4 Ctrl Fw/JEN1 Ctrl Rv. The *URA3* knockout cassette was amplified from pUG-*hphNT1* using primers URA3 KO Fw and URA3 KO Rv and transformed to IMK302, resulting in IMK322. Correct integration of the knockout cassette was confirmed using primer combinations URA3 Ctrl Fw/hphNT1 Ctrl Rv and hphNT1 Ctrl Fw/URA3 Ctrl Rv. The *KanMX4* and *hphNT1* markers in IMK322 were removed using the Cre/loxP system with pSH47 [31], resulting in IMK338. Correct marker removal was confirmed using primer combinations JEN1 Ctrl Fw/JEN1 Ctrl Rv and URA3 Ctrl Fw/URA3 Ctrl Rv.

The first *ADY2* knockout cassette was amplified from pUG-*hphNT1* using primers ADY2 KO Fw A and ADY2 KO Rv B and transformed to IMK338, IMW004 and IMW005, resulting in IMK341, IMW032 and IMW033 respectively. Correct integration of the knockout cassette was confirmed using primer combinations ADY2 Ctrl Fw/hphNT1 Ctrl Rv and hphNT1 Ctrl Fw/ADY2 Ctrl Rv. The second *ADY2* knockout cassette was amplified from pUG-*natNT2* using primers ADY2 KO Fw B and ADY2 KO Rv B and transformed to IMW032, resulting in IMW040. Correct integration of the knockout cassette was confirmed using primer combinations ADY2 Ctrl Fw/natNT2 Ctrl Rv and natNT2 Ctrl Fw/ADY2 Ctrl Rv. The third *ADY2* knockout cassette was amplified from pUG66 using primers ADY2 KO Fw C and ADY2 KO Rv C and transformed to IMW040, resulting in IMW041.

Correct integration of the knockout cassette was confirmed using primer combinations ADY2 Ctrl Fw/ble Ctrl Rv and ble Ctrl Fw/ADY2 Ctrl Rv. Presence or absence of the *ADY2* gene was confirmed using primers ADY2 inside Fw and ADY2 inside Rv. The *ADY2* gene including promoter and terminator was amplified from genomic DNA of CEN.PK113-7D, IMW004 and IMW005 using primers ADY2p Fw and ADY2t Rv and the resulting PCR products were cloned into pCR-BLUNT II-TOPO using Gateway Technology (Invitrogen, Carlsbad, USA), resulting in pUD151, pUD153 and pUD152 respectively. The *ADY2* alleles including promoter and terminator were subsequently cloned into pRS416 via the *BamH*I and *Xba*I sites, resulting in pUDC011, pUDC015 and pUDC012 respectively. pRS416, pUDC011, pUDC012 and pUDC015 were transformed into IMK341 and selected on a synthetic medium without uracil, resulting in IMZ271, IMZ272, IMZ273 and IMZ276.

Molecular biology techniques

PCR amplification was performed using Phusion Hot Start High Fidelity Polymerase (Finnzymes, Espoo, Finland) according to manufacturer's instructions in a Biometra TGradient Thermocycler (Biometra, Gottingen, Germany). DNA fragments were separated on a 1% (w/v) agarose (Sigma, St. Louis, USA) gel in 1xTAE (40 mM Tris-acetate pH 8.0 and 1 mM EDTA). Isolation of fragments from gel was performed with the Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, USA). Restriction endonucleases (New England Biolabs, Beverly, USA) and DNA ligases (Roche, Basel, Switzerland) were used according to manufacturer's instructions. Transformation and amplification of plasmids was performed in *E.coli* One Shot TOP10 competent cells (Invitrogen, Carlsbad, USA) according to manufacturer's instructions. Plasmids were isolated from *E. coli* with the Sigma GenElute Plasmid Miniprep Kit (Sigma, St. Louis, USA). DNA constructs were routinely sequenced by Baseclear BV (Baseclear, Leiden, the Netherlands).

Laboratory evolution of strains IMW004 and IMW005

Glycerol stocks of IMK302 were used to inoculate two 500-ml shake flasks containing 100 ml of synthetic medium [60] with 5 $g \cdot l^{-1}$ L-lactic acid (Fluka 09578) and 25 mM MES buffer. The pH of the medium was set to 5.0 with 2 M KOH and 2 M H_2SO_4 prior to autoclaving (121 °C, 20 min). When growth was

observed, a small aliquot (0.1-2 ml) was transferred to a new flask. After 10 transfers, the culture was plated on agar plates containing 20 $g \cdot l^{-1}$ glucose, 10 $g \cdot l^{-1}$ Bacto yeast extract and 20 $g \cdot l^{-1}$ Bacto peptone to obtain single colonies. Per evolution line, four colonies were tested for growth on lactate. One colony per evolution line was stocked and named as IMW004 and IMW005.

Cultivation and media

Shake-flask precultures for characterization experiments were inoculated with glycerol stocks and grown in synthetic medium [60] with 20 $g \cdot l^{-1}$ ethanol as carbon source.

Characterization of growth on lactate in shake flasks with an initial pH of 5.0 was performed in synthetic medium [60] with 5 $g \cdot l^{-1}$ lactate and 25 mM MES buffer, as described above. Cells growing exponentially on ethanol were used to inoculate 500-ml shake flasks containing 100 ml medium and incubated in an Innova incubator shaker (New Brunswick Scientific, Edison, USA) at 200 rpm and 30 °C.

Aerobic batch cultures for transcriptome analysis were carried out at 30 °C in 2 litre laboratory fermentors (Applikon, Schiedam, the Netherlands) with a working volume of 1 litre. Synthetic medium [60] was supplemented with 20 $g \cdot l^{-1}$ lactic acid (Fluka 09578) as sole carbon source. The pH of this medium was set to 5.0 before autoclaving (121 °C, 20 min). Antifoam Emulsion C (Sigma, St. Louis, USA) was autoclaved separately (121 °C, 20 min) as a 20% (w/v) solution and added to a final concentration of 0.2 $g \cdot l^{-1}$. The culture pH was maintained at 5.0 by automatic addition of 2 M KOH and 2 M H_2SO_4. Cultures were stirred at 800 rpm and sparged with 500 ml $air \cdot min^{-1}$. The precultures were grown on ethanol as described above. Six hours before inoculation of the batch cultures, 25 mM lactate was added to the preculture to induce lactate metabolism. Samples for RNA extraction were taken during the exponential growth phase at a cell dry weight concentration of 3-4 $g \cdot l^{-1}$ and a lactate concentration of 5-7 $g \cdot l^{-1}$. Sequential batch cultures for characterization of growth rate and biomass yield were run in the same setup as described above, but with 10 $g \cdot l^{-1}$ lactate to prevent possible nutrient limitations above 5 $g \cdot l^{-1}$ cell dry weight. The first batch was inoculated with a preculture growing exponentially on ethanol, as described above. To increase reproducibility of the results, a sequential batch reactor (SBR) was used and the third cycle was sampled [3].

Growth assays on agar plates

Growth on ethanol, lactate and pyruvate was tested by spotting 10 µl of serial dilutions of a culture growing exponentially on ethanol, on synthetic medium agarose (1% w/v) plates supplemented with 25 mM MES and either 5 $g \cdot l^{-1}$ ethanol, lactate or pyruvate. Before spotting, the cultures were first washed 2 times and diluted to the appropriate cell concentration with synthetic medium [60]. Pictures were taken after 6 days incubation at 30 °C.

Analytical methods

Optical density at 660 nm was measured with a Libra S11 spectrophotometer (Biochrom, Cambridge, UK). Culture dry weights were determined via filtration of appropriate sample volumes (10-20 ml) over dry preweighed nitrocellulose filters (Gelman Laboratory, Ann Arbor, USA) with a pore size of 0.45 µm. After removal of the medium, the filters were washed twice with demineralized water, dried in a microwave oven for 20 min at 350 W and weighed. Culture supernatants were obtained after centrifugation of the broth. Supernatants and media were analyzed via HPLC using an Aminex HPX-87H ion exchange column operated at 60 °C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 $ml \cdot min^{-1}$. Off-gas was first cooled in a condenser (2 °C) and dried with a Perma Pure Dryer (Permapure, Toms River, USA). CO_2 concentrations in the off-gas were measured with a NGA 2000 Rosemount gas analyzer (Rosemount Analytical, Orrville, USA).

Microarrays and analysis

Sampling of cells from the batch cultures and total RNA extraction was performed as described previously [2]. Probe preparation and hybridization to Affymetrix Genechip® microarrays were performed according to Affymetrix's instructions. The one-cycle eukaryotic target labelling assay was used, starting with 15 µg of total RNA. The quality of total RNA, cRNA and fragmented cRNA was checked using the Agilent BioAnalyzer 2100 (Agilent Technologies). Results for each strain were obtained from 2 independent culture replicates. The Significance Analysis of Microarrays (SAM, version 1.12) [53] add-in to Microsoft Excel was used for comparison of replicate array experiments using a fold-change threshold of two and an expected false discovery rate of 5%. Transcript data have been deposited in the Genome Expression Omnibus

database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE31634.

Whole-genome sequencing and analysis

DNA of the reference strain CEN.PK113-7D was prepared as described previously [11]. A library of 200-bp fragments was created and sequenced paired-end using the Illumina Solexa system, generating ca. 56 million 36-bp paired reads. These reads were mapped to the genome of *S. cerevisiae* strain S288C (downloaded 2010/07/21 from SGD [1]) using the Burrows-Wheeler Alignment tool BWA [39]. Subsequently, a consensus sequence was generated using SAMtools' pileup2fq [40] with default parameters, except for the minimum depth and root-mean-square mapping quality, which were set to 10 and 25 respectively. Consensus bases not passing the filtering thresholds, mostly in repetitive regions, were replaced by 'N'. This consensus was used as CEN.PK113-7D reference genome.

Genomic DNA from IMW004 and IMW005 was isolated using the Qiagen 100/G kit (Qiagen, Hilden, Germany). A library of 200-bp genomic fragments was created and sequenced paired-end (50-bp reads) using an Illumina HiSeq 2000 sequencer by Baseclear BV (Baseclear, Leiden, the Netherlands). The individual reads were mapped onto the genome of CEN.PK113-7D, using BWA [39] and further processed using SAMtools [40]. Single-nucleotide variations and small insertions and deletions were extracted from the mapping using SAMtools' varFilter. Default settings were used, except that the maximum read depth was set to 300x (-D300) and the minimum small insertions and deletions score for single-nucleotide variations filtering was set to 60 (-G60). To minimize false-positive mutation calls, custom Perl scripts and Microsoft Excel were used for further mutation filtering. First, mutation calls containing ambiguous bases in either reference or mapping consensus were filtered out. Second, only the single-nucleotide variations with a quality of at least 20 and small insertions and deletions with a quality of at least 50 were kept. Variant quality is defined as the Phred-scaled probability that the mutation call is incorrect [22]. Third, mutations with a depth of coverage less than 10x were discarded. Fourth, insertion and deletion mutation calls were only kept when at least 85% of the reads spanning the location confirmed the insertion or deletion. Fifth, small insertions and deletions that were close to an 'N' in the reference were removed, since this would complicate correct alignments and introduce

false-positive mutation calls. For small insertions and deletions a window size of 40 bp was used, for single-nucleotide variations a window size of 5 bp. Copy number variation was analyzed using CNV-seq [68].

Pulsed-field gel electrophoresis and southern blotting

Chromosomes were separated using the CHEF yeast genomic DNA Plug Kit (170-3593, Bio-Rad, Richmond, USA) according to manufacturer's instructions and subsequently transferred onto Hybond-N^+ nylon membranes (RPN303, Amersham Biosciences, Piscataway, USA). Southern blotting, signal generation and signal detection was performed using the Gene Images AlkPhos Kit, CPD Star detection reagent and Hyperfilm ECL (RPN 3680, RPN3682 and 28-9068, Amersham Biosciences, Piscataway, USA). The DNA probe used for southern blotting was amplified from CEN.PK113-7D genomic DNA with primers ADY2 inside Fw and ADY2 inside Rv (Table 2.3).

Results

Laboratory evolution of Jen1p-independent growth on lactate

To test whether laboratory evolution and subsequent analysis of the underlying mutations is a powerful strategy to identify alternative lactate transporters in *Saccharomyces cerevisiae*, a *jen1*Δ knockout strain was constructed and evolved for growth on lactate. Hitherto, Jen1p is the only efficient importer of lactate in *S. cerevisiae* described in literature [12]. Consistent with earlier reports, *S. cerevisiae* IMK302 (*jen1*Δ) did not show growth on plates with lactate after 6 days, whereas the reference strain CEN.PK113-7D (*JEN1*) grew normally (Figure 2.1). In shake-flask cultures with 5 $g \cdot l^{-1}$ lactate at pH 5 the same results were obtained (μ<0.001 h^{-1} for IMK302 (*jen1*Δ)), but after prolonged incubation (5-15 days) growth was observed in two independent experiments. To select for faster growth, small aliquots of the shake-flask cultures were transferred to fresh medium for 9 consecutive times. After ca. 100 generations in 10 shake-flask cultures, single-colony isolates were obtained by plating on non-selective agar plates containing glucose, yeast extract and peptone. Per evolution line, four single-colony isolates were tested for growth on lactate and yielded essentially the same growth rates as the mixed population from which they were isolated. One single-colony isolate from each evolution experiment was chosen for further characterisation. These resulting evolved strains were named IMW004

and IMW005 and grew at maximum specific growth rates of 0.14 and 0.18 h^{-1}, respectively, on 20 $g \cdot l^{-1}$ lactate in aerobic bioreactor batch cultures (pH 5.0).

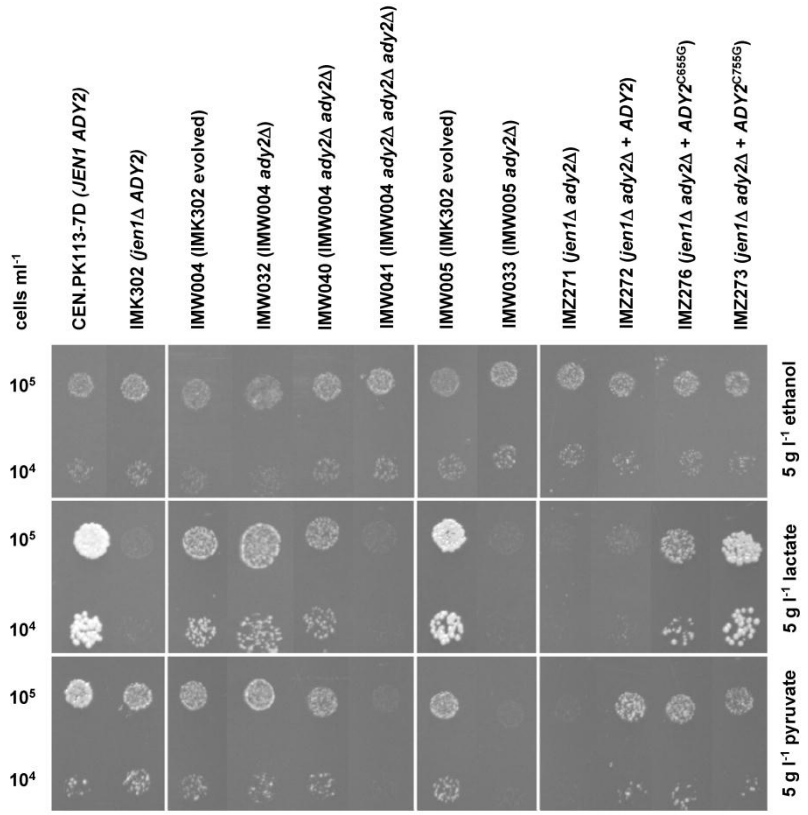

Figure 2.1 Growth of *S. cerevisiae* strains with different combinations of *JEN1* and *ADY2* alleles on ethanol, lactate and pyruvate. 10 µl of a suspension with the indicated cell concentration was spotted onto synthetic medium agarose (1% w/v) plates at pH 5 with 5 $g \cdot l^{-1}$ ethanol (upper panel), 5 $g \cdot l^{-1}$ lactate (middle panel) or 5 $g \cdot l^{-1}$ pyruvate (lower panel) as the sole carbon source. Pictures were taken after 6 days of incubation at 30 °C.

Mutation analysis through whole-genome DNA sequencing and transcriptome analysis

Two techniques were used to study the molecular basis of Jen1p-independent growth on lactate in the evolved strains IMW004 and IMW005: transcriptome analysis with DNA microarrays and whole-genome DNA resequencing. In view

of the highly reproducible growth conditions and the ability to control the specific growth rate, chemostat cultures are normally the preferred cultivation type for comparative transcriptome analysis [16]. However, chemostat cultures growing on lactate demonstrated persistent oscillations which precluded steady-state analysis (data not shown). Therefore, cell samples for transcriptome analysis were obtained from aerobic bioreactor batch cultures grown on 20 $g \cdot l^{-1}$ lactate and at pH 5.0. To minimize differences in nutrient concentration and growth conditions, all cultures were sampled at a biomass dry weight concentration between 3 and 4 $g \cdot l^{-1}$, corresponding to 5-7 $g \cdot l^{-1}$ residual lactate. The average deviation of the mean of all genes with mRNA expression levels above background (in this case 12) was between 16 and 21%, which was only slightly higher than chemostat-based transcriptome analysis (10-15%) [8]. When comparing the transcriptome data of CEN.PK113-7D (*JEN1*) with IMW004 and IMW005 (both *jen1*Δ evolved), only a very small number of genes (11 for IMW004 and 18 for IMW005) were expressed at different levels based on the statistical criteria applied in this study (absolute fold difference ≥ 2; false discovery rate 5%, see Materials and methods) (Figure 2.2). In addition to *JEN1* (deleted in the evolved strain) and the introduced *KANMX4* dominant marker gene, the general amino acid permease *GAP1* was the only gene that was differentially expressed in both evolution lines IMW004 and IMW005. However, the transcript levels of *GAP1* were only 2.6- and 2.0- fold higher in IMW004 and IMW005, respectively, than in the reference strain CEN.PK113-7D.

In parallel, genomic DNA of IMW004 and IMW005 (both *jen1*Δ evolved) was sequenced using Illumina technology, yielding ca. 12.8 million 50-bp reads for IMW004 and ca. 13.7 million reads for IMW005. The average depth of coverage was 51.5-fold for IMW004 and 56.6-fold for IMW005. The sequencing reads were mapped against the genome sequence of the reference strain CEN.PK113-7D, which was constructed by mapping CEN.PK113-7D sequencing reads to the S288C sequence. This resulted in 20 and 16 single-nucleotide variations for IMW004 and IMW005, respectively. No insertions or deletions were found. The set of relevant mutations was reduced to 7 for IMW004 and 10 for IMW005 (Table 2.4) by eliminating single-nucleotide variations outside coding regions, since no links were found with mRNA expression levels of adjacent genes. Nucleotide changes that only result in different codon usage for the same amino acid might affect translation

efficiency and were therefore not discarded. Interestingly, several of the remaining single-nucleotide variations were either identical in both IMW004 and IMW005 or different mutations had occurred in the same genes (Table 2.4). Such mutations might (i) present genes that are relevant for Jen1p-independent growth on lactate, (ii) have accumulated during construction of the *jen1*Δ strain and have been present before the start of the evolution experiment or (iii) represent errors in the reference sequence.

Table 2.4 Mutation calls inside genes in IMW004 and IMW005 identified via whole-genome sequencing.

Gene	Description	Nucleotide change	Amino acid change
Genes mutated in both IMW004 and IMW005			
YAT1	Mitochondrial carnitine acetyltransferase	C75A	None
ADY2	Acetate transporter	C655G (IMW005)	Leu219Val (IMW005)
ADY2	Acetate transporter	C755G (IMW004)	Ala252Gly (IMW004)
BSC1	Hypothetical protein	T553A	Thr185Ser
FLO11	Glycoprotein (flocculin)	G1716A (IMW005)	None
FLO11	Glycoprotein (flocculin)	C1743T (IMW005)	None
FLO11	Glycoprotein (flocculin)	G2361A	None
DDC1	DNA damage checkpoint protein	G1513C	Ala505Pro
Genes mutated only in IMW004			
YNL179C	Hypothetical protein	T156A	Leu52Phe
CAF120	Part of transcriptional regulatory complex	G1689A	None
Genes mutated only in IMW005			
FIT1	Cell wall mannoprotein	A1067G	Val356Ala
PAU2	Member of seripauperin multigene family	T318C	None
YHR028W-A	Hypothetical protein	T217C	Ser73Phe

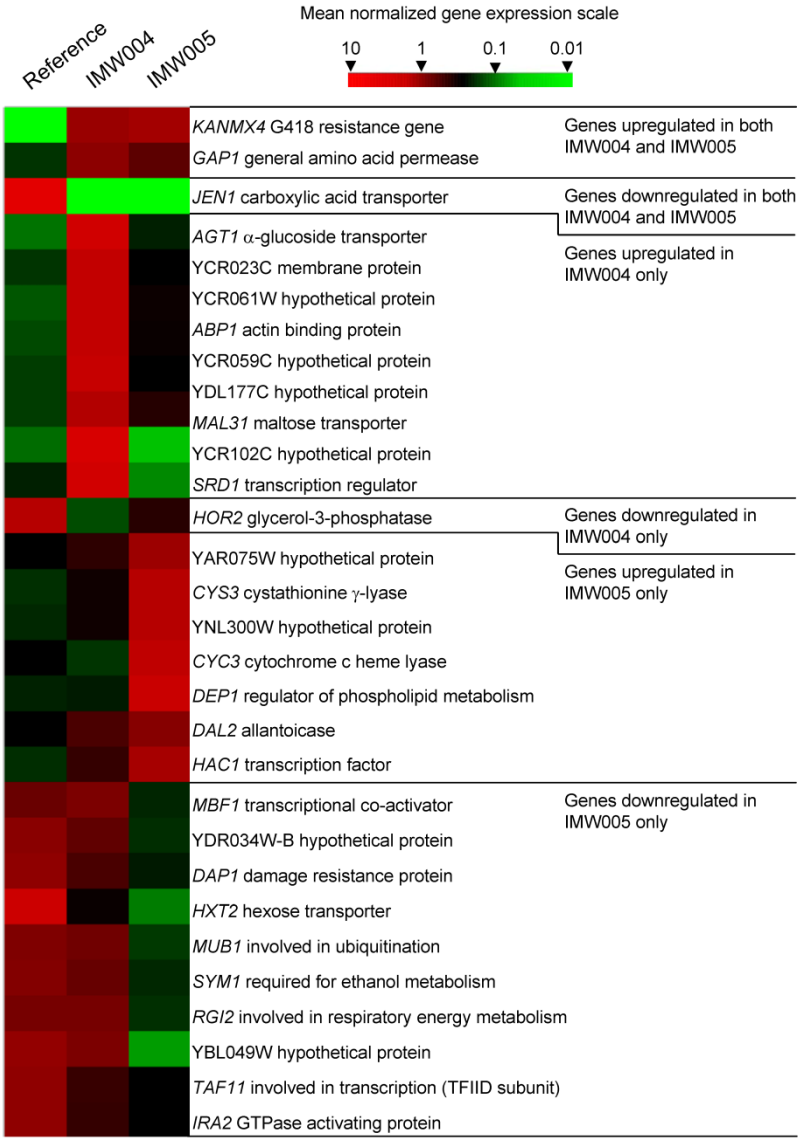

Figure 2.2 Differentially expressed genes in IMW004 and IMW005 (both *jen1*Δ evolved) compared to the reference strain CEN.PK113-7D (*JEN1*) during exponential growth on 20 $g \cdot l^{-1}$ lactate at pH 5.0. Expression data were compared using a fold-change threshold of two and an estimated false discovery rate of 5%.

Rearrangement of chromosome III

Rearrangement of larger DNA fragments, including deletions and duplications, is a well-known phenomenon during (yeast) evolution [19, 20, 66, 67]. To analyze these larger structural variations, the genome-wide mRNA expression levels and depth of coverage of DNA sequencing were plotted according to their chromosomal location. Interestingly, both the mRNA expression levels and depth of coverage of DNA sequencing showed ^{2}log ratios between 1 and 2 for a group of genes on chromosome III, when comparing IMW004 and CEN.PK113-7D (Figure 2.3). Detailed investigation of the depth of coverage of DNA sequencing indicated a duplication of chromosome III between 83 kb and 126 kb, including the centromere, and a triplication between 126 kb to the end of the right arm (Figure 2.4, panel A). These observations led to the hypothesis that, in addition to chromosome III an additional, pseudopalindromic isochromosome III was formed containing the centromere and two copies of the right arm of chromosome III (Figure 2.4, panel B). Karyotyping confirmed the formation of an additional chromosome with a size of ca. 475 kb (Figure 2.5), which corresponds well to the proposed rearrangement (Figure 2.4, panel C).

To exactly determine the chromosomal rearrangement, the DNA sequences 5 kb up- and downstream of the observed breakpoints (i.e. 78-88 kb and 121-131 kb) were aligned with each other. The highest homology was observed between the Ty1-type long terminal repeat retrotransposons *YCLWΔ15* and *YCRCΔ6*, with 96% homology over a region of 176 bp. To verify whether crossover took place between *YCLWΔ15* and *YCRCΔ6* as proposed in Figure 2.4 (panel C), primer combinations were designed (P5 and P6) that should give a 3041 bp PCR product if crossover had indeed taken place between *YCLWΔ15* and *YCRCΔ6*. In line with the proposed mechanism of rearrangement, the expected 3 kb PCR product was obtained from IMW004, but not from CEN.PK113-7D genomic DNA. Sequencing of this DNA fragment showed that crossover took place between the last 98 homologous bp of *YCLWΔ15* and *YCRCΔ6*.

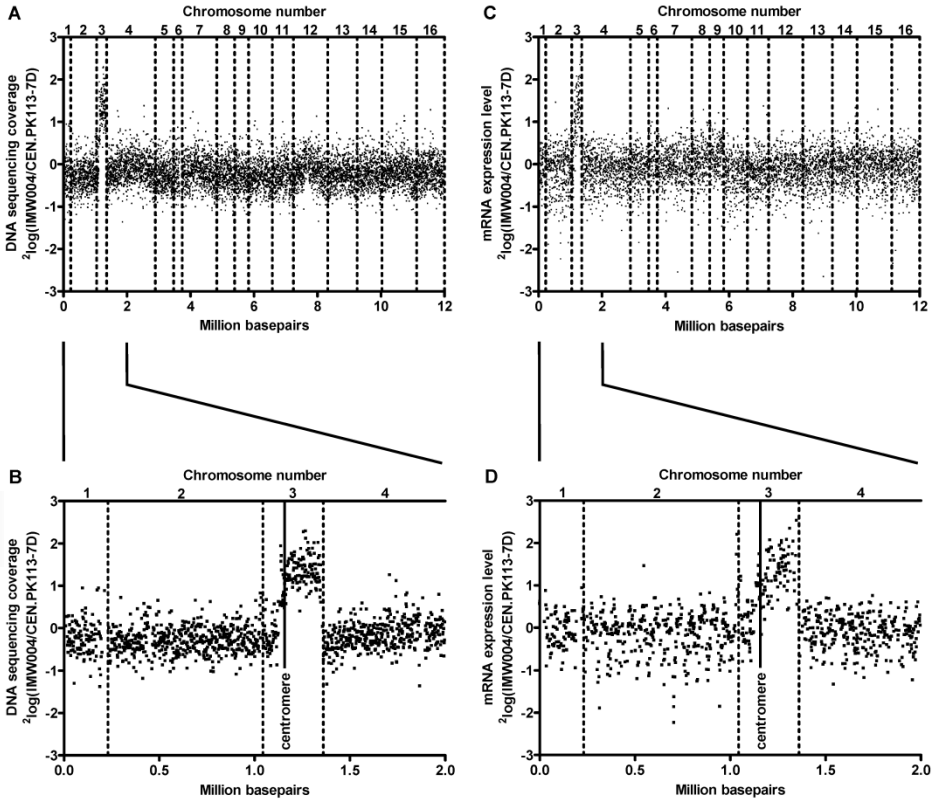

Figure 2.3 Comparison of depth of coverage of DNA sequencing (panel A and B) and mRNA expression levels (panel C and D) of CEN.PK113-7D (*JEN1*) and IMW004 (*jen1*Δ evolved). Values were plotted according to their chromosomal location. Chromosome numbers are indicated above the panels. Depth of coverage of DNA sequencing was averaged and plotted per 1500 bp. For mRNA expression levels, only genes with an expression level above 12 were considered.

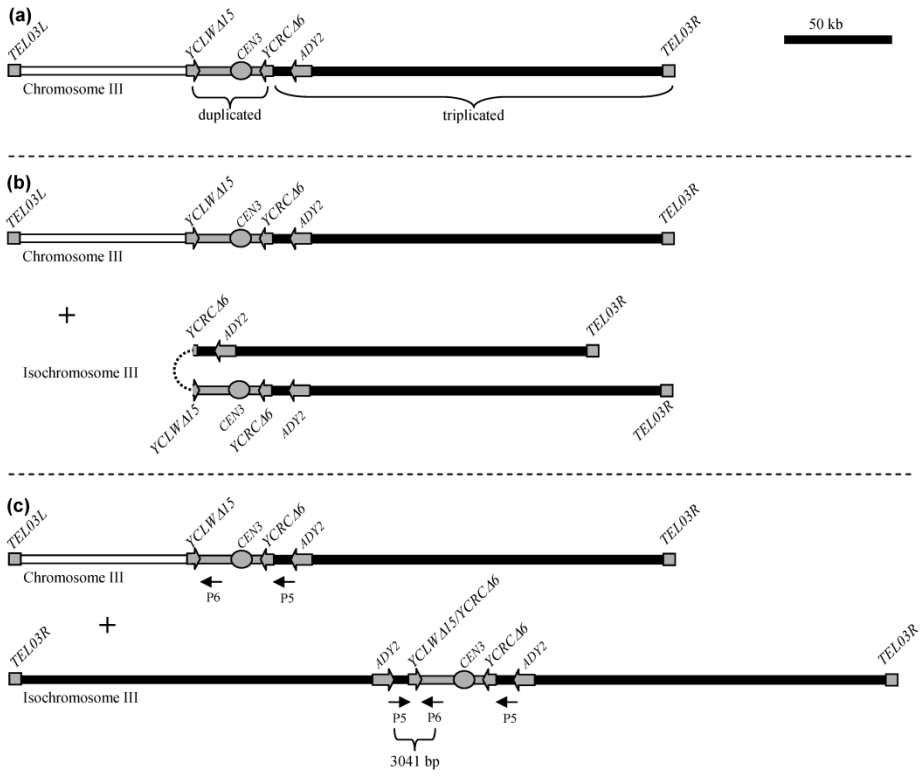

Figure 2.4 Analysis of chromosomal rearrangements in IMW004. **(a)** Copy number estimates on chromosome III based on depth of coverage of DNA sequencing and mRNA expression levels. **(b)** Crossover between homologous regions of *YCLWΔ15* and *YCRCΔ6*, **(c)** resulting in the formation of an additional, pseudopalindromic isochromosome III, containing two extra copies of *ADY2*^{C755G}. Indicated are the binding sites of primers P5 and P6, which were used to confirm that crossover took place as proposed. Duplicated parts of chromosome III are indicated in grey; triplicated regions in black. Relevant features are indicated, but not drawn to scale.

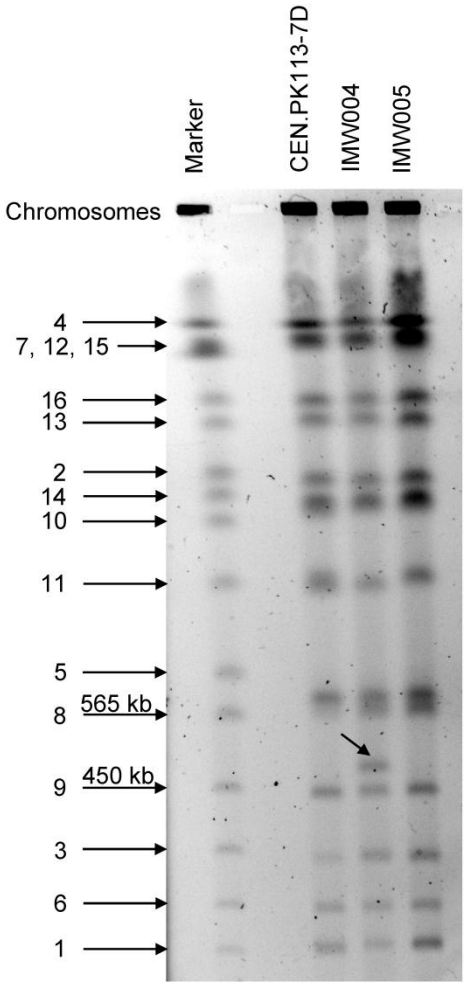

Figure 2.5 Pulsed-field gel electrophoresis (PFGE) analysis of the reference strain CEN.PK113-7D and the evolved strains IMW004 and IMW005. Chromosome numbers and sizes are indicated on the left side. YNN295 (Bio-Rad #170-3605) was used as marker. The additional chromosome (ca. 475 kb) present in IMW004 is indicated by the diagonal arrow.

Verification of the identified mutations in the acetate transporter *ADY2*

The *ADY2* gene, which encodes an acetate transporter [43], contained mutations that resulted in amino acid changes in both independently evolved strains (Table 2.4) and is located on the triplicated region of chromosome III of IMW004. To analyze whether the different copies of *ADY2* in IMW004 all

contained the same mutation, the individual sequencing reads mapping to *ADY2* were analyzed. At all positions a constant signal was observed, showing that IMW004 carried three identical copies of *ADY2*^{C755G}. *ADY2* was not amongst the genes whose mRNA expression levels were significantly different from those in the reference strain (1.4-fold in IMW004 and 0.6-fold IMW005).

To investigate the physiological relevance of the mutation (C755G; Ala252Gly) and the apparent triplication of *ADY2* in IMW004, knockout strains were constructed. Diagnostic PCR on genomic DNA of the single, double and triple knockout strains (IMW032, IMW040 and IMW041) only showed absence of *ADY2* in the triple knockout strain (IMW041), thereby confirming that IMW004 contained three copies of *ADY2*^{C755G}. Only the triple knockout strain was unable to grow on lactate (Figure 2.1), confirming that Ady2p^{Ala252Gly} was essential for lactate import in the evolved strain IMW004.

To analyze the effect of *ADY2*^{C755G} gene dosage on the growth rate on lactate, aerobic shake flask experiments were performed with 5 g·l^{-1} lactate at pH 5. IMW004 (3 copies of *ADY2*^{C755G}) grew at a growth rate of 0.10±0.01 h^{-1}; IMW032 (2 copies) at 0.09±0.00 h^{-1}, IMW040 (1 copy) at 0.07±0.00 h^{-1} and IMW041 (0 copies) did not grow (μ<0.001 h^{-1}) on lactate.

To analyze whether the identified mutation (C655G; Leu219Val) in *ADY2* could also fully explain the acquired phenotype in IMW005, a knockout of *ADY2*^{C655G} was constructed. IMW033 (IMW005 *ady2*Δ) was not able to grow on lactate, thereby confirming the crucial role of Ady2p^{Leu219Val} in lactate import in IMW005 (Figure 2.1).

Reverse metabolic engineering of mutated *ADY2* alleles

To verify whether mutation of *ADY2* alone is sufficient to allow for growth on lactate, the mutated alleles were introduced into a non-evolved strain background (*jen1*Δ *ady2*Δ). IMZ271 (*jen1*Δ *ady2*Δ) and IMZ272 (*jen1*Δ *ady2*Δ + *ADY2*) did not grow on agar plates with 5 g·l^{-1} lactate after 6 days of incubation, whereas IMZ273 (*jen1*Δ *ady2*Δ + *ADY2*^{C755G}) and IMZ276 (*jen1*Δ *ady2*Δ + *ADY2*^{C655G}) did grow on these plates (Figure 2.1). This confirmed that these alleles can confer the ability to transport lactate. IMZ271 (*jen1*Δ *ady2*Δ) could still grow on acetate (data not shown), which prevented kinetic analysis of acetate transport mediated by the mutated Ady2p alleles. Interestingly, IMZ271 (*jen1*Δ *ady2*Δ) could not grow on pyruvate, whereas IMZ272 (*jen1*Δ *ady2*Δ + *ADY2)*, IMZ273 (*jen1*Δ *ady2*Δ + *ADY2*^{C755G}) and IMZ276 (*jen1*Δ *ady2*Δ +

ADY2^{C655G}) could, showing that both the non-mutated and the mutated Ady2p alleles can function as efficient pyruvate importers in this strain background (Figure 2.1), in contrast to previous reports on other *S. cerevisiae* strains [4].

Subsequently, two strains expressing the native *ADY2* allele were compared. IMK302 (*jen1*Δ *ADY2*), which contains the native *ADY2* gene on chromosome III, did not grow on lactate (μ<0.001 h^{-1}) in shake-flask cultures with 5 $g \cdot l^{-1}$ lactate at pH 5. The fact that IMZ272 (*jen1*Δ *ady2*Δ + *ADY2*), containing the native *ADY2* under control of its own promoter on a centromeric vector, grew very slowly on lactate (μ_{max} ca. 0.02 h^{-1}) demonstrates that even non-mutated Ady2p can transport lactate, although at a very low rate. The observed differences in growth may be due to differences in mRNA expression level between chromosomal (IMK302) and centromeric (IMZ272) expression. As a result, it might be that in IMZ272, but not in IMK302, the lactate consumption rate is high enough to overcome the maintenance-ATP requirement and thus allow for (slow) growth [9, 45].

To further characterize the mutated alleles of *ADY2*, the reverse engineered strains IMZ273 (*jen1*Δ *ady2*Δ + *ADY2*^{C755G}) and IMZ276 (*jen1*Δ *ady2*Δ + *ADY2*^{C655G}) were tested in aerobic sequential batch fermentations with 10 $g \cdot l^{-1}$ lactate at pH 5.0. The reference strain CEN.PK113-7D (*JEN1 ADY2*) was used for comparison, since in this strain the large majority of lactate transport proceeds via Jen1p and not via Ady2p (Figure 2.1). Both IMZ273 (*jen1*Δ *ady2*Δ + *ADY2*^{C655G}) and IMZ276 (*jen1*Δ *ady2*Δ + *ADY2*^{C755G}) grew rapidly on lactate at maximum specific growth rates of 0.14±0.00 and 0.12±0.00 h^{-1}, respectively. In cultures of strain IMZ276, the Ady2p^{Ala252Gly}-dependent lactate consumption rate decreased towards the end of the fermentation, indicating a low affinity for lactate (Figure 2.6). The biomass yield on lactate of IMZ273 (0.37±0.00 $g \cdot g^{-1}$; *jen1*Δ *ady2*Δ + *ADY2*^{C655G}) was considerably higher than those of IMZ276 (0.30±0.01 $g \cdot g^{-1}$; *jen1*Δ *ady2*Δ + *ADY2*^{C755G}) and CEN.PK113-7D (0.26±0.01 $g \cdot g^{-1}$; *JEN1 ADY2*).

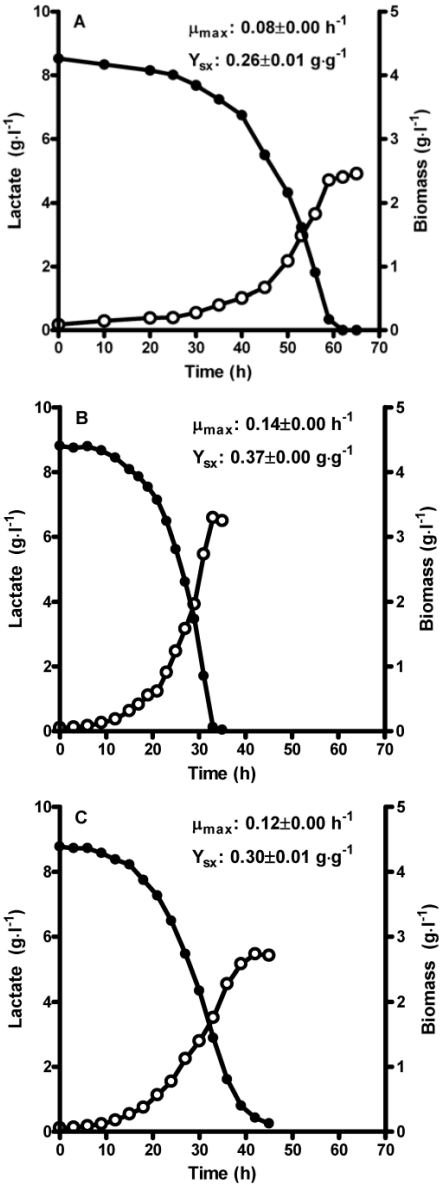

Figure 2.6 Growth of CEN.PK113-7D (*JEN1 ADY2*, panel A), IMZ273 (*jen1*Δ *ady2*Δ + *ADY2*^{C655G}, panel B) and IMZ276 (*jen1*Δ *ady2*Δ + *ADY2*^{C755G}, panel C) during the third cycle of an aerobic sequential batch fermentation on lactate at pH 5.0. The results shown are from one representative experiment. Averages and mean deviations for the maximum specific growth rates (μ_{max}) and biomass yields (Y_{sx}) were obtained from independent duplicate experiments.

Discussion

Ady2p as lactate transporter

No protein structure of Ady2p or close homologues is available and therefore the effect of the mutations on the structure and function of Ady2p remains uncertain. However, the observation that single-nucleotide mutations turned the acetate transporter Ady2p into an efficient lactate transporter provides valuable information for future structure-function analysis. In both evolution lines, the identified mutations were located in putative transmembrane-domains [1] and resulted in amino acids that are one carbon atom smaller than the original amino acid (alanine to glycine in IMW004 and leucine to valine in IMW005). Since lactate ($C_3H_6O_3$) is structurally similar, but one carbon atom larger than acetate ($C_2H_4O_2$), this suggests that extra space is created in the translocation site of Ady2p.

According to the literature, Jen1p catalyzes an electroneutral lactate-proton symport, which is energetically equivalent to diffusion of the undissociated acid [12, 14]. Complete aerobic dissimilation of 1 lactate then yields 7 ATP: 6 from oxidative phosphorylation at an *in vivo* P/O ratio of 1.0 [7, 55] and 1 from substrate-level phosphorylation in the tricarboxylic-acid cycle. To generate and maintain a proton-motive force, the plasma-membrane H^+-ATPase in yeast exports (only) a single proton for each ATP that it hydrolyzes [56, 63]. Lactate transport mechanisms that result in outward translocation of a proton and/or inward translocation of a negative charge can save ATP equivalents that would otherwise be used for the build-up or maintenance of the proton-motive force. These savings can therefore not exceed the *in vivo* activity of the plasma-membrane H^+-ATPase. In this way, lactate import via a lactate anion uniport or via a lactate-proton antiport mechanism could increase the apparent ATP yield from lactate dissimilation to 8 or 9 $mol \cdot mol^{-1}$, corresponding to increases in the biomass yield on lactate of 14 % and 29 %, respectively. The reverse engineered strain IMZ273 ($Ady2p^{Leu219Val}$) displayed a 24±5% higher biomass yield on lactate than IMZ276 ($Ady2p^{Ala252Gly}$) and a 41±3% higher biomass yield than the reference strain CEN.PK113-7D (Figure 2.6), in which lactate import primarily proceeds via Jen1p (Figure 2.1). Taking into account that an additional ca. 5% of the biomass yield increase can be explained due to higher specific growth rates of the evolved strains, which causes a lower impact of maintenance-energy requirements [9, 45], these biomass yield values might be

consistent with a different mode of energy coupling of lactate transport. Further analysis of the energy coupling of lactate transport by these Ady2p isoforms, involving *in vitro* studies will provide valuable insights into the energetics of organic acid transport in yeast and its impact on growth energetics.

Chromosomal rearrangement

Chromosomal rearrangement is a well-known phenomenon during (yeast) evolution [19, 67] and occurs often by recombination of Ty1-type retrotransposons (reviewed by [26]). The occurrence of a ca. 475 kb isochromosome III in strain IMW004 shows how even a short (ca. 100 generations) period of selective growth can select for a massive chromosomal rearrangement, in this case resulting in duplication of around 25 genes and triplication of about 100 genes. In addition to enhancing performance under the conditions that led to their selective advantage, such rearrangements may be 'hopeful monsters' that open up novel evolutionary paths via specialization of duplicated genes [51].

In-depth analysis of the sequencing reads showed that all three copies of *ADY2* in IMW004 contained the same nucleotide change (C755G), which suggests that the mutation in *ADY2* occurred before the triplication event. This indicates that first a gain of function mutation in *ADY2* provided the cells with a selective advantage over the parental strain. The subsequent retrotransposon-mediated triplication of *ADY2*^{C755G} apparently gave sufficient evolutionary advantage to compensate for the burden of an additional chromosome. In line with this hypothesis, a strain derived from IMW004 containing only one copy of *ADY2*^{C755G} (IMW040) showed a 30% lower growth rate on lactate. The observation that the second and third copy of *ADY2*^{C755G} did not lead to a two- and three-fold higher growth rates can be explained by saturation of transcription, possibly due to a limited availability of transcription factors that regulate *ADY2* expression, such as Cat8p, Adr1p and Snf1p [13]. Indeed, the *ADY2* mRNA expression level of IMW004 (three *ADY2*^{C755G} copies) was only 40% higher than that of CEN.PK113-7D (one *ADY2* copy). Simulation of the evolution experiment, based on the observed cell densities and the relatively small difference in growth rate between strains expressing one (0.07 h^{-1}) and three (0.10 h^{-1}) *ADY2*^{C755G} copies, indicated that the formation of the isochromosome III occurred early in the evolution experiment before the first

transfer. Accordingly, no further increase in the growth rate on lactate was observed in the remaining 9 shake flasks of the evolution experiment.

Functional analysis through laboratory evolution and reverse engineering

Laboratory evolution combined with molecular analysis of the evolved strains enabled the successful identification of alternative lactate importer genes in *S. cerevisiae*. Although this strategy can only be used in cases where the gene function can be linked to a selective advantage, it can be very useful in situations where only a low residual enzyme activity is observed for which the genetic determinants are unknown, such as for glucose transport or disaccharide hydrolysis in mutant strains of *S. cerevisiae* in which all known genes for these activities have been deleted [18, 64]. In addition, this approach can contribute genes and proteins with new, interesting properties for metabolic engineering. For example, the possible different energy coupling of Ady2p^{Leu219Val} identified in this study may be highly relevant to metabolic engineering of *S. cerevisiae* for lactic acid production [58, 59].

Elucidation of the genetic basis of the acquired phenotype is a crucial step in laboratory evolution-based functional analysis, which in this study was approached by a combination of transcriptome analysis and whole-genome resequencing. Subsequent verification via knockout studies and reverse metabolic engineering showed that the relevant changes could be identified via whole-genome resequencing, but not via transcriptome analysis. In yeast, only few studies have been performed to identify the relevant genetic changes and reverse engineer them into a non-evolved strain background. When studies on both prokaryotic and eukaryotic laboratory-evolved strains are considered, a clear picture emerges that, in many studies, transcriptome analysis did not allow rapid identification of relevant genetic changes that had been selected for in laboratory evolution experiments [23-25, 34, 46, 57], even when combined with metabolome analysis [61] or proteome analysis [38]. In contrast, studies in which the genetic basis for the acquired phenotype was elucidated and reconstructed via reverse metabolic engineering, analyzed the DNA level via whole-genome resequencing or hybridization of genomic DNA to microarrays [5, 15, 29, 32, 33, 37, 52]. These observations support the view that analysis of the genome sequence rather than the transcriptome is the most promising first-line analytical approach for elucidating relevant changes that occurred during evolution. Transcriptome analysis can then be used as a follow-up in cases where whole-

genome resequencing does not generate obvious and productive leads. In this respect, mRNA sequencing is an interesting development that allows simultaneous measurement of mRNA expression levels and identification of mutations inside coding regions [42, 62]. Independent of the technique used, analyzing multiple independently evolved strains strongly facilitates dissection of relevant and random genetic changes.

Acknowledgments

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Chapter 3

Increasing free-energy (ATP) conservation in maltose-grown *Saccharomyces cerevisiae* by expression of a heterologous maltose phosphorylase

Stefan de Kok, Duygu Yilmaz, Erwin Suir, Jack T. Pronk, Jean-Marc Daran and Antonius J.A. van Maris

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Abstract

Increasing free-energy (ATP) conservation from the conversion of substrate into product is crucial for further development of many biotechnological processes. In theory, replacing the hydrolysis of disaccharides by a phosphorolytic cleavage reaction provides an opportunity to increase the ATP yield on the disaccharide. To test this concept, we first deleted the native maltose metabolism genes in *Saccharomyces cerevisiae*. The knockout strain showed no maltose-transport activity and a very low residual maltase activity (0.03±0.01 μmol·mg protein^{-1}·min^{-1}). Expression of a maltose phosphorylase gene from *Lactobacillus sanfranciscensis* and the *S. cerevisiae MAL11* maltose transporter gene resulted in relatively slow growth ($\mu_{aerobic}$ 0.09±0.03 h^{-1}). Co-expression of *Lactococcus lactis* β-phosphoglucomutase accelerated maltose utilization via this route ($\mu_{aerobic}$ 0.21±0.01 h^{-1}, $\mu_{anaerobic}$ 0.10±0.00 h^{-1}). Replacing maltose hydrolysis with phosphorolysis increased the anaerobic biomass yield on maltose in anaerobic maltose-limited chemostat cultures by 26%, thus demonstrating the potential of phosphorolysis to improve free-energy (ATP) conservation of disaccharide metabolism in industrial microorganisms.

Introduction

Showcases such as the biotechnological production of 1,3-propanediol with *Escherichia coli* [37] and the anti-malarial precursor artemisinic acid with *Saccharomyces cerevisiae* [42] demonstrate the maturation of metabolic engineering. Introduction and optimization of heterologous enzymes and pathways through metabolic modelling, synthetic biology and high-throughput screening allows production of a wide range of biological molecules [15, 36]. The development of efficient microorganisms that closely approximate maximum theoretical product yields requires cellular homeostasis of the redox cofactors (e.g. NAD(P)(H)) and conservation of free energy (e.g. in the form of ATP) for growth, cellular maintenance and/or product formation.

Aerobic respiration enables redox cofactor regeneration for product pathways that would otherwise result in a surplus of NAD(P)H [21, 28] and can also provide the cells with ATP via oxidative phosphorylation. However, aeration of industrial-scale fermentations is expensive due to the cost of stirring, air compression and cooling. In addition, aeration results in dissimilation of part of the substrate to CO_2, thereby decreasing the product yield. Therefore, where possible, it would be beneficial to produce commodity chemicals through redox-neutral pathways, as is the case for alcohol, lactic acid and many metabolic engineering targets, which allows industrial production under anaerobic conditions.

In the conversion of glucose via the classical Embden-Meyerhof-Parnas glycolytic pathway, substrate-level phosphorylation results in the net formation of 2 ATP for each molecule of glucose converted. A challenging situation arises for products of interest whose formation from glucose does not result in a net formation of ATP when produced through redox-neutral routes under anaerobic conditions. In many such cases, the ATP formed in glycolysis by substrate-level phosphorylation may subsequently be used for carboxylation reactions [59, 60], product export [53] or the formation of acyl-CoA esters [46, 52]. Production of lactic acid by metabolically engineered *S. cerevisiae* strains is an illustrative example. The conversion of glucose to 2 molecules of lactic acid yields 2 ATP. However, in *S. cerevisiae* export of lactic acid is hypothesized to require 1 ATP per lactic acid. This results in a 'zero-ATP pathway' from glucose to extracellular lactic acid. This phenomenon presents an intrinsic limitation for efficient production of lactic acid under anaerobic conditions and at low pH

[53], since, without a net formation of ATP, cells cannot grow or fulfil the free-energy requirements for cellular maintenance.

Increasing free-energy (ATP) conservation from the conversion of substrate into product is of major importance for such 'zero-ATP pathways'. This study will explore the possibilities to increase free-energy (ATP) conservation during growth on disaccharides. In many industrial microorganisms, disaccharides are hydrolyzed (Reaction 3.1), which dissipates the free energy available from this cleavage. Alternatively, disaccharides can be cleaved with inorganic phosphate (Reaction 3.2), thereby increasing free-energy conservation of this reaction through the direct formation of phosphorylated intermediates [61]:

$$\text{disaccharide} + H_2O \rightarrow \text{monosaccharide} + \text{glucose} \quad (3.1)$$

$$\text{disaccharide} + P_i \rightarrow \text{monosaccharide} + \text{glucose-1-phosphate} \quad (3.2)$$

Disaccharide phosphorylases are described for sucrose, maltose, cellobiose, trehalose and lactose [3, 6, 14, 16]. Functional expression of heterologous cellobiose phosphorylase in *S. cerevisiae* has recently been demonstrated [44]. The formed glucose-1-phosphate can be converted by a phosphoglucomutase into glucose-6-phosphate, which is further metabolized via glycolysis. As a result no ATP is hydrolyzed in the hexokinase reaction to convert glucose to glucose-6-phosphate. This modification increases the net ATP yield by 1 ATP per disaccharide molecule.

In this study, growth of *S. cerevisiae* on maltose was used as a model. The genetic structure of maltose metabolism in *S. cerevisiae* is well described [39]. It consists of several *MAL* loci, which contain a maltose permease (*MALx1*), a maltase (*MALx2)* and a regulator (*MALx3).* These *MAL* loci are located subtelomerically and the number of *MAL* loci is strain dependent [38]. Additionally, Mph2p and Mph3p can transport maltose [13]. In *S. cerevisiae*, maltose is imported via a proton-symport mechanism. The imported proton has to be exported to maintain intracellular pH homeostasis. In *S. cerevisiae*, the plasma-membrane H^+-ATPase expels 1 proton per ATP hydrolyzed. Maltose is subsequently hydrolyzed by the intracellular maltases into two molecules of glucose (ΔG_0` –15.5 kJ·mol$^{-1}$), which can be converted through normal sugar metabolism. Anaerobic conversion of maltose to ethanol therefore yields 3 ATP [51, 56]. An anaerobic catabolic route that uses maltose phosphorolysis ($\Delta G_0$` +5.5 kJ·mol^{-1}) instead of maltose hydrolysis would generate 4 instead of 3 ATP per maltose. Theoretically, this ATP yield can be further increased to 5 ATP per

maltose (2.5 ATP per hexose unit) when the proton coupling of maltose transport is abolished.

The goal of the present study is to investigate whether maltose hydrolysis in *S. cerevisiae* can be replaced by phosphorolytic cleavage and to quantitatively analyze the impact of such a replacement on free-energy (ATP) conservation in anaerobic maltose-grown cultures. To this end, the native maltose metabolism of *S. cerevisiae* was deleted and a bacterial maltose phosphorylase was introduced. Subsequently, enzyme activities, growth rates and biomass yields on maltose were analyzed in batch and chemostat cultures.

Materials and methods

Strains and maintenance

The *S. cerevisiae* strains used and constructed in this study (Table 3.1) are congenic members of the CEN.PK family [18, 50] and contain a constitutive *MAL* activator gene *MAL2-8*^{C} [19]. Stock cultures were grown at 30 °C in shake flasks containing 100 ml synthetic medium [55] with 20 $g \cdot l^{-1}$ glucose as carbon source. After overnight growth, 20% (v/v) glycerol was added and 1-ml aliquots were stored at –80 °C.

Plasmid construction

The plasmids and primers used in this study are listed in Table 3.2 and 3.3, respectively. The *hphNT1* marker was cloned from pFA6a-*hphNT1* [27] to pUG6 [24] via the *Sac*I and *Bgl*II restriction sites, thereby replacing the *KanMX4* marker, resulting in pUG-*hphNT1*. The *ADH1* terminator was amplified from plasmid pRW231 [57] using primers ADH1t Fw and ADH1t Rv and cloned into p426GPD [35] via the *Kpn*I and *Spe*I restriction sites, thereby replacing the *CYC1* terminator, resulting in pUD63. *MAL12* was amplified from CEN.PK113-7D genomic DNA with primers MAL12 Fw and MAL12 Rv and cloned into pUD63 via the *Hind*III and *Spe*I sites, resulting in pUDE44. *mapA* was amplified from *Lactobacillus sanfranciscensis* (DSM 20451) genomic DNA using primers mapA Fw and mapA Rv and cloned into pENTR/D-TOPO using Gateway Technology (Invitrogen, Carlsbad, USA), resulting in pUD37. The *mapA* gene was recombined into pvv214 [54] via the LR reaction using Gateway Technology (Invitrogen, Carlsbad, USA), resulting in pUDE31. The *mapA* gene of plasmid pUD37 was cloned into pUD63 via the *Hind*III and *Spe*I

sites, resulting in pUDE45. *pgmB* was ordered as a synthetic gene at Baseclear B.V. (Leiden, the Netherlands). The gene was codon-optimized for *S. cerevisiae* (according to [22]) and was flanked by attB-sites and *Pst*I and *BamH*I restriction sites 5' of the gene and *Hind*III and *Sal*I sites 3' of the gene (GenBank accession no. JF514919). The *pgmB* gene was first cloned to pDONR221 via the BP reaction using Gateway Technology (Invitrogen, Carlsbad, USA), resulting in pUD88 and then cloned to pAG426GPD [2] via the LR reaction using Gateway Technology (Invitrogen, Carlsbad, USA), resulting in pUDE60. The gene was cloned into pUD63 via the *Spe*I and *Xho*I sites, resulting in pUDE63. The *mapA* gene and *PGK1* promoter and *CYC1* terminator were amplified from pUDE31 using primers PGK1p Fw and CYC1t Rv and cloned into pUDE63 using the *Age*I and *Sgf*I restriction sites, resulting in pUDE82. A multiple cloning site was constructed using primers Mcs Fw and Mcs Rv and cloned into pENTR/D-TOPO, resulting in pUD65. The *MAL11* gene was amplified from CEN.PK113-7D genomic DNA using primers MAL11 Fw and MAL11 Rv and cloned into pUD65 using the *Nde*I and *Fse*I sites and recombined into pAG305GPD [2], resulting in pUDI35.

Table 3.1 *Saccharomyces cerevisiae* strains used in this study.

Strain	Relevant genotype
CEN.PK113-7D	MATa *MAL1x MAL2x MAL3x LEU2 URA3 MAL2-8^C*
CEN.PK102-3A	MATa *MAL1x MAL2x MAL3x leu2-112 ura3-52 MAL2-8^C*
IMK289 (*mal*Δ *mph*Δ)	CEN.PK102-3A *mal11-mal12::loxP mal21-mal22::loxP mal31-mal32::loxP mph2::loxP mph3::loxP*
IMX020	IMK289 pRS405 (*LEU2*)
IMX032	IMK289 pUDI35 (*LEU2 MAL11*)
IMZ133	IMK289 pRS405 (*LEU2*) pUD63 (*URA3)*
IMZ135	IMK289 pRS405 (*LEU2*) pUDE44 (*URA3 MAL12*)
IMZ229	IMK289 pUDI35 (*LEU2 MAL11*) pUD63 (*URA3)*
IMZ199	IMK289 pUDI35 (*LEU2 MAL11*) pUDE44 (*URA3 MAL12*)
IMZ203	IMK289 pUDI35 (*LEU2 MAL11*) pUDE45 (*URA3 mapA*)
IMZ226	IMK289 pUDI35 (*LEU2 MAL11*) pUDE82 (*URA3 mapA pgmB*)

Table 3.2 Plasmids used in this study.

Plasmid	Characteristic
pENTR/D-TOPO	Gateway entry clone
pDONR221	Gateway entry clone
pUG6	PCR template for *loxP-KanMX4-loxP* cassette
pFA6a-*hphNT1*	Plasmid with *hphNT1* marker
pSH47	Centromeric plasmid, *URA3*, P_{GAL1}-*Cre*-T_{CYC1}
pRW231	2 µm ori, *URA3*, P_{TPI1}-*xylA*-T_{CYC1} P_{TDH3}-*araA*-T_{ADH1} P_{HXT7}-*araD*-T_{PGI1}
p426GPD	2 µm ori, *URA3*, P_{TDH3}-T_{CYC1}
pvv214	2 µm ori, *URA3,* P_{PGK1}-*ccdB*-T_{CYC1}
pAG426GPD	2 µm ori, *URA3,* P_{TDH3}-*ccdB*-T_{CYC1}
pAG305GPD	Integration plasmid, *LEU2*, P_{TDH3}-*ccdB*-T_{CYC1}
pRS405	Integration plasmid, *LEU2*
pUG-*hphNT1*	PCR template for *loxP-hphNT1-loxP* cassette
pUD37	Gateway entry clone, *mapA*
pUD63	2 µm ori, *URA3*, P_{TDH3}-T_{ADH1}
pUD65	Gateway entry clone, multiple cloning site
pUD88	Gateway entry clone, *pgmB*
pUDE31	2 µm ori, *URA3,* P_{PGK1}-*mapA*-T_{CYC1}
pUDE44	2 µm ori, *URA3*, P_{TDH3}-*MAL12*-T_{ADH1}
pUDE45	2 µm ori, *URA3*, P_{TDH3}-*mapA*-T_{ADH1}
pUDE60	2 µm ori, *URA3,* P_{TDH3}-*pgmB*-T_{CYC1}
pUDE63	2 µm ori, *URA3,* P_{TDH3}-*pgmB*-T_{ADH11}
pUDE82	2 µm ori, *URA3,* P_{TDH3}-*pgmB*-T_{ADH1} P_{PGK1}-*mapA*-T_{CYC1}
pUDI35	Integration plasmid, *LEU2*, P_{TDH3}-*MAL11*-T_{CYC1}

Strain construction

Transformations of *S. cerevisiae* were carried out as described previously [20]. Gene deletions and subsequent marker removal were performed using the loxP-marker-loxP/Cre recombinase system [24], using pUG6 and pUG-*hphNT1* as templates for PCR amplification of the knockout cassettes. Selection of mutants was performed on either 200 mg·l^{-1} G418 or 200 mg·l^{-1} hygromycin B. Primers used for amplifications of knockout cassettes are listed in Table 3.3. During the construction of the *S. cerevisiae* strain deleted for the native maltose metabolism, the maltose transporter (*MALx1*) and maltase (*MALx2*) within the same locus were deleted with one knockout cassette, starting in the double auxotrophic CEN.PK102-3A strain. The order of knockout was: *MAL11* and *MAL12*,

MAL31 and *MAL32*, *MAL21* and *MAL22*, followed by *MPH2* and *MPH3*. Knockouts were confirmed by southern blotting on separated chromosomes using a *MAL32* probe, amplified from CEN.PK113-7D genomic DNA with primers MAL32 probe Fw and MAL32 probe Rv, or a *MPH2/3* probe, amplified from CEN.PK113-7D genomic DNA with primers MPH2/3 probe Fw and MPH2/3 probe Rv. The resulting maltose transporter and maltase knockout strain was designated IMK289. Plasmids pRS405 [45] and pUDI35 were linearized with *BstE*II and transformed into IMK289, resulting in IMX020 and IMX032 respectively. IMX020 was transformed with pUD63 and pUDE44, resulting in IMZ133 and IMZ135 respectively. IMX032 was transformed with pUDE44, pUDE45, pUDE82 and pUD63, resulting in IMZ199, IMZ203, IMZ226 and IMZ229 respectively (Table 3.1).

Molecular biology techniques

PCR amplification was performed using Phusion Hot Start High Fidelity Polymerase (Finnzymes, Espoo, Finland) according to manufacturer's instructions in a Biometra TGradient Thermocycler (Biometra, Gottingen, Germany). DNA fragments were separated on a 1% (w/v) agarose (Sigma, St. Louis, USA) gel in 1xTAE (40 mM Tris-acetate pH 8.0 and 1 mM EDTA). Isolation of fragments from gel was performed with the Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, USA). Restriction endonucleases (New England Biolabs, Beverly, USA and Promega, Madison, USA) and DNA ligases (Roche, Basel, Switzerland) were used according to manufacturer's instructions. Transformation and amplification of plasmids was performed in *E. coli* One Shot TOP10 Competent cells (Invitrogen, Carlsbad, USA) according to manufacturer's instructions. Plasmids were isolated from *E. coli* with the Sigma GenElute Plasmid Miniprep Kit (Sigma, St. Louis, USA). DNA constructs were routinely sequenced by Baseclear BV (Baseclear, Leiden, the Netherlands). Chromosomes were separated using the CHEF yeast genomic DNA Plug Kit (170-3593, Bio-Rad, Richmond, USA) according to manufacturer's instructions and subsequently transferred onto Hybond-N^+ nylon membranes (RPN303, Amersham Biosciences, Piscataway, USA). Southern blotting, signal generation and signal detection was performed using the Gene Images AlkPhos Kit, CPD Star detection reagent and Hyperfilm ECL (RPN 3680, RPN3682 and 28-9068, Amersham Biosciences, Piscataway, USA).

Table 3.3 Primers used in this study.

Primer name	Sequence (5'→3')
Primers for knockouts	
MAL1 KO Fw	CTTAACATTTATCAGCTGCATTTAATTCTCGCTGTTTTATGCTTGAGG ACTGACTCCAATGGCAGGTAATGTACGCAGCTGAAGCTTCGTACGC
MAL1 KO Rv	GCACTAATTTTATTTGACGAGGTAGATTCTACCTTCCCATGGTTTCAA AACTCTGGCATAGGCCACTAGTGGATCTG
MAL3 KO Fw A	CATTTGTTCACAACAGATGAGGTGTTTCGCCCTTCATCTACCACAGAA GTTTCCACAATTAGACCTCCCTACAGTGCAGCTGAAGCTTCGTACGC
MAL3 KO Rv A	CTCTGGAGGAAACGTCAGTATCATCATAATTTCCAAGAATAAAAGAT AAAGAAGCGCATAGGCCACTAGTGGATCTG
MAL3 KO Fw B	CCAAATCTTCCTTCGGATCTTTAACATTAATTTCTGCAGCTGCTGCTT TGAACAGAAGCCCATGTGGTGACAGCTGAAGCTTCGTACGC
MAL3 KO Rv B	ATAAAAGATAAAGAAGCACCTTCTCTTGGGAGGCTGAATTCAATTTC TTCGACCCTTCTGAGACGGTAGTGGCATAGGCCACTAGTGGATCTG
MPH2/3 KO Fw A	GTTTGTAATTCTTAGAGGCCTGTTCTTGGAATTATTATGCAAAGATT ACCATCACCAGCTGAAGCTTCGTACGC
MPH2/3 KO Rv A	ACAAATCTACGTGTATTATACTCCGTAACATGTAGAGTAAATACCATA GTTACCTGCATAGGCCACTAGTGGATCTG
MPH2/3 KO Fw B	AAATCTAACACAAATAGAGATACAGGTCTTGTAAAGCCATTACCTAG CTATGAAACAGCTGAAGCTTCGTACGC
MPH2/3 KO Rv B	TACCTGTGTCGATAAATGTTCATTAGCTCATAAGTGATGGGATACAT TGCTATTCGCATAGGCCACTAGTGGATCTG
Primers for probes	
MAL32 Probe Fw	GCAGAAGGGCAATCTTTGAAAGTG
MAL32 Probe Rv	AGCAGCAAACAGCGTCTTGTC
MPH2/3 Probe Fw	GGATACAAGAGCGCCAAGCGATAG
MPH2/3 Probe Rv	GTCTTTCCGGCAGTTTCTGGTAGG
Primers for cloning	
mapA Fw	CACCGTTTAAACACTAGTGGAGAATATCATGAAGCGAATTTTTGAAG
mapA Rv	GGCCGTTTAAACAAGCTTCATCTTAGGCCTCCAAAGTTAGC
ADH1t Fw	CACTCGAGGGTTGGCATGACTAAGGCATTC
ADH1t Rv	CTGGTACCCGATACCGGTGATGGCGATCGCGTGTGGAAGAACGATT ACAACAGG
MAL12 Fw	CACCACTAGTCATAAATGACTATTTCTGATCATCCAGAAACAG
MAL12 Rv	GCAAGCTTGCCGGCACTAATTTTATTTGACGAG
Mcs Fw	CACCCATATGATATGTTTAAACGCATGCTGAGCATCGCCATGG
Mcs Rv	GCGGCCGGCCGCATCACCGGTGCGATCCATGGCGATGCTCAGC
MAL11 Fw	CACCAGTCATATGGTATAATATGAAAAATATCATTTCATTGGTAAGC AAGAAG
MAL11 Rv	GCATTAGGCCGGCCCTTAACATTTATCAGCTGCATTTAATTCTCG
PGK1p Fw	GCACCGGTACGTCGTACGCGACTCTTTTCTTCTAACCAAGGG
CYC1t Rv	CAGCGATCGCCTTCAAAGCTTGCAAATTAAAGCC

Cultivation

Characterization in shake flasks was performed in a medium containing per litre 20 g maltose (Molekula, Dorset, UK), 6.6 g K_2SO_4, 3 g KH_2PO_4, 2.3 g urea and 0.5 g $MgSO_4 \cdot 7\ H_2O$ and trace elements and vitamins as described previously [55]. The pH of the medium was set to 5.0 with 2 M KOH and 2 M H_2SO_4 prior to filter sterilization. Cultures were prepared by inoculating 100 ml medium in a 500-ml shake flask with 1-ml frozen stock culture and incubated in an Innova incubator shaker (New Brunswick Scientific, Edison, USA) at 200 rpm and 30 °C. Optical density at 660 nm was measured in regular time intervals with a Libra S11 spectrophotometer (Biochrom, Cambridge, UK). Anaerobic batch and chemostat fermentations were carried out at 30 °C in 2 litre laboratory bioreactors (Applikon, Schiedam, the Netherlands) with a working volume of 1 litre. A synthetic medium with 25 $g \cdot l^{-1}$ maltose as the sole carbon and energy source was used for all chemostats [55]. The medium was supplemented with the anaerobic growth factors ergosterol (10 $mg \cdot l^{-1}$) and Tween 80 (420 $mg \cdot l^{-1}$) dissolved in ethanol. Antifoam Emulsion C (Sigma, St. Louis, USA) was autoclaved separately (121 °C, 20 min) as a 20% (w/v) solution and added to a final concentration of 0.2 $g \cdot l^{-1}$. The culture pH was maintained at 5.0 by the automatic addition of 2 M KOH. Cultures were stirred at 800 rpm and sparged with 500 $ml \cdot min^{-1}$ nitrogen (<10 ppm oxygen). To minimize diffusion of oxygen, the bioreactors were equipped with Norprene tubing and Viton O-rings and the medium vessels were flushed with nitrogen gas. The anaerobic growth rate was obtained by plotting the CO_2 production rate, which is stoichiometrically coupled to growth, on a logarithmic scale and calculating the slope of the linear part of the graph.

Plate growth assays

Growth on glucose and maltose was tested by spotting 10 µl of serial dilutions of cultures, growing exponentially on glucose, onto synthetic medium agarose (1% w/v) plates with either 20 $g \cdot l^{-1}$ glucose or 20 $g \cdot l^{-1}$ maltose. To remove the glucose present as a contaminant in the maltose that was used (Molekula, Dorset, UK), IMZ133 – which can grow on glucose, but not on maltose – was cultivated in a double concentrated synthetic medium with 40 $g \cdot l^{-1}$ maltose. After 3 days of incubation, HPLC analysis showed that all glucose was removed and all maltose was retained. The broth was centrifuged, filter-sterilized using 0.2 µm filters (Nalgene, New York, USA) and mixed with 2% (w/v) agarose to

pour the spot plates. Before spotting, the cultures were first washed 3 times with synthetic medium [55] to remove residual glucose.

Analytical methods

Chemostat cultures were assumed to be in steady state when, after at least five volume changes, the culture dry weight and carbon dioxide production rates changed by less than 4% over 2 volume changes. Steady-state samples were taken between 10-15 volume changes after inoculation to avoid possible evolutionary adaptation during long-term cultivation. Culture dry weights were determined via filtration of 20-ml samples over dry preweighed nitrocellulose filters (Gelman Laboratory, Ann Arbor, USA) with a pore size of 0.45 µm. After removal of the medium, the filters were washed twice with demineralized water, dried in a microwave oven for 20 min at 350 W and weighed. Culture supernatants were obtained after centrifugation of chemostat broth. Supernatants and media were analyzed via HPLC using an Aminex HPX-87H ion exchange column operated at 60 °C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml·min^{-1}. Samples for residual maltose were taken as described previously [32]. Ethanol concentrations were corrected for evaporation as described previously [23]. Off-gas was first cooled in a condenser (2 °C) and dried with a Perma Pure Dryer (Permapure, Toms River, USA). CO_2 concentrations in the off-gas were measured with a NGA 20000 Rosemount gas analyzer (Rosemount Analytical, Orrville, USA). Culture viability was assayed using the *Funga*light CFDA, AM/propidium iodide yeast vitality kit (Invitrogen, Carlsbad, USA) on a Cell Lab Quanta™ SC MPL flow cytometer (Beckman Coulter, Woerden, the Netherlands). Viability (metabolic activity) was calculated as the number of $CFDA^+$ and PI^- cells divided by the total number of cells.

Enzyme activity measurements

For preparation of cell extracts, culture samples (around 62.5 mg cell dry weight) were harvested from exponentially growing shake-flask cultures on 20 g·l^{-1} maltose. The samples were centrifuged (4 °C, 10 min at 5000 g), washed twice with freeze buffer (10 mM potassium phosphate, pH 7.5 and 2 mM EDTA), washed once with disruption buffer (100 mM potassium phosphate, pH 7.5, 2 mM $MgCl_2$ and 1 mM DTT) and resuspended in 2-ml disruption buffer. Cells were disrupted in a FastPrep 120 (Qbiogene, Carlsbad, USA) by shaking 1-ml aliquots with 0.75 g glass beads (425–600 µm, Sigma-Aldrich,

Zwijndrecht, the Netherlands) four times 20 s at power level 6, with 1 min cooling on ice in between. Non-disrupted cells and cell debris were removed by centrifugation (4 °C, 20 min at 36000 g) and the supernatant was used as the cell extract for enzyme assays. Protein levels in cell extracts were determined using a Lowry assay [30]. For IMZ229, cells were harvested from shake-flask cultures with 20 $g \cdot l^{-1}$ ethanol and 20 $g \cdot l^{-1}$ maltose. Cell extracts for β-phosphoglucomutase activity measurements were prepared from fresh, non-frozen cultures to avoid the observed instability of the enzyme upon freezing. Maltase activity was measured at 30 °C by monitoring the reduction of $NADP^+$ at 340 nm in a 1-ml reaction mixture containing 100 mM maltose, 50 mM imidazole-HCl buffer (pH 6.6), 1 mM $NADP^+$, 12.5 mM $MgCl_2$, 1 mM ATP, 3.5 units hexokinase and 3.5 units glucose-6-phosphate dehydrogenase. The reaction was started by the addition of 1-100 µl cell extract. Maltose phosphorylases and β-phosphoglucomutase activities were measured at 30 °C by monitoring the reduction of $NADP^+$ at 340 nm in a 1-ml reaction mixture containing 100 mM MES buffer (pH 7.0), 25 mM potassium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM $NADP^+$, 0.01 mM glucose-1,6-bisphosphate, 3.5 units glucose-6-phosphate dehydrogenase and 1-100 µl cell extract. The reaction was started by the addition of 100 mM maltose. For determination of the individual maltose phosphorylases or β-phosphoglucomutase activity, 3.5 units of commercial β-phosphoglucomutase (Sigma, St. Louis, USA) or maltose phosphorylases (Sigma, St. Louis, USA) were added to the reaction mixture respectively. An extinction coefficient of 6.3 mM^{-1} was assumed for NADPH. Reaction rates were proportional (within 25%) to the amounts of cell extract added.

Results

Deletion of native maltose transporters and maltases

To enable a systematic analysis of the bioenergetic impact of genetic modifications in maltose metabolism, we first constructed a strain platform in which the native maltose metabolism genes of *S. cerevisiae* (consisting of the *MAL* loci, *MPH2* and *MPH3*) were deleted. The number of *MAL* loci is strain dependent [38]. Southern blot analysis on separated chromosomes using a *MAL32* probe showed that CEN.PK102-3A contained three *MAL* loci (*MAL1x MAL2x* and *MAL3x*, respectively on chromosomes VII, III and II;

data not shown). The maltose transporters (*MALx1*) and maltases (*MALx2*) are adjacent genes, which enabled deletion of both genes in one transformation using a single knockout cassette. Using the Cre/loxP system [24] for removal of dominant marker genes, the maltose transporter (*MALx1*) and maltase (*MALx2*) genes of the three *MAL* loci were deleted, followed by *MPH2* and *MPH3*. Southern blot analysis with *MAL32* and *MPH2/3* probes confirmed that all known maltases and maltose transporters were successfully deleted (data not shown). The resulting strain (*mal11-mal12*Δ *mal21-mal22*Δ *mal31-mal32*Δ *mph2*Δ *mph3*Δ) was named IMK289.

Characterization of the maltose knockout (*mal*Δ *mph*Δ) strain

To determine whether the *S. cerevisiae* genome encodes additional maltose transporters and maltases, growth tests were employed with strains derived from IMK289 (*mal*Δ *mph*Δ). To analyze residual activity of maltose transport and hydrolysis, the maltase-encoding *MAL12* gene and the maltose transporter gene *MAL11* (also known as *AGT1*) were re-introduced in IMK289, either alone or in combination. The resulting prototrophic strains were tested on agarose plates for growth on glucose and maltose as the sole carbon source. The reference strain CEN.PK113-7D (Mal^+) and IMZ199 (*mal*Δ *mph*Δ *MAL11 MAL12*) grew on maltose, whereas IMZ133 (*mal*Δ *mph*Δ), IMZ135 (*mal*Δ *mph*Δ *MAL12*) and IMZ229 (*mal*Δ *mph*Δ *MAL11*) did not show growth on maltose after 3 days of incubation (Figure 3.1). To quantify the specific growth rates on maltose, the strains were incubated in shake flasks with maltose as the sole carbon source (Table 3.4). Under these conditions, CEN.PK113-7D (Mal^+, 0.34±0.00 h^{-1}) and IMZ199 (*mal*Δ *mph*Δ *MAL11 MAL12*, 0.24±0.00 h^{-1}) grew fast. IMZ133 (*mal*Δ *mph*Δ) and IMZ135 (*mal*Δ *mph*Δ *MAL12*) did not show any growth during 7 days (μ<0.001 h^{-1}), but IMZ229 (*mal*Δ *mph*Δ *MAL11*) grew slowly (0.007±0.001 h^{-1}) in liquid medium (Table 3.4). These results showed that all maltose-transport activity was removed and that a low residual maltose-hydrolyzing activity was still present in the maltose knockout strain.

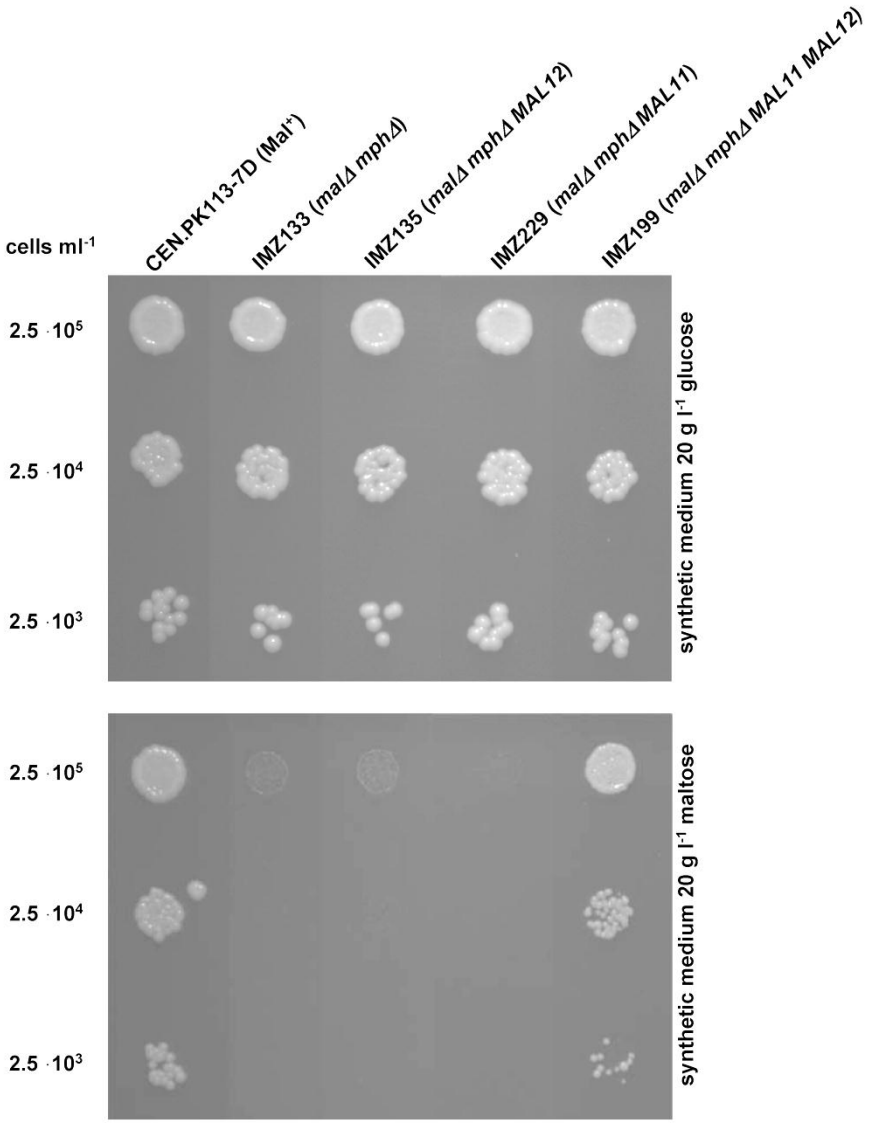

Figure 3.1 Growth on glucose and maltose of CEN.PK113-7D (reference) and four maltose knockout strains (*mal*Δ *mph*Δ) complemented with the maltose transporter gene *MAL11* and/or the maltase gene *MAL12*. 10 µl of culture was spotted onto synthetic medium agarose (1% w/v) plates at pH 5 with 20 g·l^{-1} glucose (upper panel) or 20 g·l^{-1} maltose (lower panel) as the sole carbon source. Pictures were taken after 3 days of incubation at 30 °C.

To quantify the remaining maltase activity in the maltose knockout strain background, cell extracts were prepared from IMZ229 (*mal*Δ *mph*Δ *MAL11*). Because IMZ229 only grew very slowly on maltose (0.007±0.001 h^{-1}), a different carbon source was chosen that supports faster growth. Considerations in choosing another carbon source were that maltose metabolism is repressed by glucose and induced by maltose [10], even though the CEN.PK strains contain a constitutive *MAL* activator gene *MAL2-8^C* [19]. Therefore, cells extracts were prepared from an IMZ229 culture grown on the non-repressing carbon source ethanol (2% w/v) in the presence of the inducer maltose (2% w/v). This revealed a maltase activity of 0.03±0.00 μmol·min^{-1}·mg $protein^{-1}$ for IMZ229 (*mal*Δ *mph*Δ *MAL11*), while CEN.PK113-7D (Mal^+) and IMZ199 (*mal*Δ *mph*Δ *MAL11 MAL12*) grown on maltose showed a maltase activity of 3.6±0.1 and 5.9±1.2 μmol·min^{-1}·mg $protein^{-1}$ respectively. Thus, the residual maltose-hydrolyzing activity of the maltose knockout strain was less than 1% of that of the reference strain.

Table 3.4 Aerobic and anaerobic growth rates on maltose of *Saccharomyces cerevisiae* strains carrying different combinations of maltase, maltose phosphorylase and β-phosphoglucomutase. Averages and mean deviations were obtained from duplicate experiments.

Strain	Relevant genotype	Growth rate (h^{-1})	
		Aerobic[a]	Anaerobic[b]
CEN.PK113-7D	*MAL1x MAL2x MAL3x MPH2 MPH3*	0.34±0.00	0.28±0.01
IMZ133	*mal*Δ *mph*Δ	<0.001	N.D.
IMZ135	*mal*Δ *mph*Δ + *MAL12*	<0.001	N.D.
IMZ229	*mal*Δ *mph*Δ + *MAL11*	0.007±0.001	N.D.
IMZ199	*mal*Δ *mph*Δ + *MAL11* + *MAL12*	0.24±0.00	0.12±0.00
IMZ203	*mal*Δ *mph*Δ + *MAL11* + *mapA*	0.09±0.03	0.03±0.01[c]
IMZ226	*mal*Δ *mph*Δ + *MAL11* + *mapA* + *pgmB*	0.21±0.01	0.10±0.00

[a]Aerobic growth rates were based on optical density measurements in shake flasks at pH 5.0 and with 20 $g \cdot l^{-1}$ maltose. A growth rate <0.001 h^{-1} means that no growth was detected during 7 days.
[b]Anaerobic growth rates were based on CO_2 production in batch fermentations at pH 5.0 and 25 $g \cdot l^{-1}$ maltose.
[c]No consistent exponential growth was observed for this culture. Average of 40 h of incubation.
N.D. not determined

Successful expression of *Lactobacillus sanfransiscensis* maltose phosphorylase

After removing virtually all maltase activity, the next goal was to express a functional maltose phosphorylase in a strain expressing the maltose transporter

gene *MAL11*. This modification should increase free-energy conservation with 1 ATP per maltose compared to normal maltose hydrolysis in *S. cerevisiae*. In literature, only two maltose phosphorylase genes have been cloned and characterized [17, 26]. The *mapA* gene from *Lactobacillus sanfranciscensis* was chosen, because of a better *S. cerevisiae* codon adaptation index (calculated according to [22]). The *L. sanfranciscensis mapA* gene was cloned behind the strong constitutive *TDH3* promoter on the multicopy plasmid pUDE45. The resulting *mapA* expressing strain IMZ203 displayed a high maltose phosphorylase activity (11.3±1.1 μmol·min^{-1}·mg protein^{-1}), while the maltose phosphorylase activity of the reference strain CEN.PK113-7D was below the detection limit (<0.005 μmol·min^{-1}·mg protein^{-1}).

Although IMZ203 contained a high maltose phosphorylase activity, its (aerobic) growth rate on maltose was relatively low (0.09±0.03 h^{-1}). The higher growth rate (0.24±0.00 h^{-1}) of the isogenic maltase expressing strain IMZ199 (*mal*Δ *mph*Δ *MAL11 MAL12*) indicated that the maltose transporter Mal11p was not rate limiting. Since the *in vitro* maltose phosphorylase activity (11.3±1.1 μmol·min^{-1}·mg protein^{-1}) of IMZ203 was even higher than the maltase activity (5.9±1.2 μmol·min^{-1}·mg protein^{-1}) of the fast-growing strain IMZ199 (0.24±0.00 h^{-1}), it is unlikely that this step controlled the specific growth rate. However, maltose phosphorylase cleaves maltose into glucose and β-glucose-1-phosphate [16], which makes β-phosphoglucomutase activity necessary to convert this β-glucose-1-phosphate into glucose-6-phosphate, which can be further catabolized via glycolysis. It was unknown whether *S. cerevisiae* contains β-phosphoglucomutase activity. To investigate this hypothesis, the β-phosphoglucomutase activity in cell extracts of CEN.PK113-7D (Mal^{+}) and IMZ203 (*mal*Δ *mph*Δ *MAL11 mapA*) was measured. In both strains, the β-phosphoglucomutase activity was below the detection limit (<0.005 μmol·min^{-1}·mg protein^{-1}).

Co-expression of *Lactococcus lactis* β-phosphoglucomutase is essential for efficient maltose utilization via maltose phosphorylase

To investigate whether β-phosphoglucomutase is the rate limiting step in the maltose phosphorylase expressing strain IMZ203, a β-phosphoglucomutase was co-expressed in this strain. In literature, several β-phosphoglucomutases have been described [31, 33, 41]. The *Lactococcus lactis pgmB* was chosen, because it has the highest reported specific activity [41]. A codon-optimized *L. lactis pgmB* and

the *L. sanfranciscensis mapA* were cloned into the multicopy plasmid pUDE82, under the control of the *TDH3* and *PGK1* promoter, respectively. The resulting strain IMZ226 showed high activities of maltose phosphorylase (0.83±0.03 μmol·min^{-1}·mg protein^{-1}) and β-phosphoglucomutase (0.58±0.04 μmol·min^{-1}·mg protein^{-1}). The difference in *in vitro* maltose phosphorylase activity in IMZ203 (0.83±0.03 μmol·min^{-1}·mg protein^{-1}) and IMZ226 (11.3±1.1 μmol·min^{-1}·mg protein^{-1}) might be due to different promoters used (P_{PGK1} and P_{TDH3}, respectively) and/or different plasmid sizes [12]. To assay each of the individual activities, an excess of the other enzyme was added. The combined enzyme activity of maltose phosphorylase and β-phosphoglucomutase was also determined in one assay. The combined enzyme activity (0.92±0.14 μmol·min^{-1} ·mg protein^{-1}) was higher than the individual β-phosphoglucomutase activity (0.58±0.04 μmol·min^{-1}·mg protein^{-1}). This might be due to alleviation of substrate inhibition of β-glucose-1-phosphate on β-phosphoglucomutase activity.

To investigate the impact of co-expression of the *L. lactis* β-phosphoglucomutase on the growth rate on maltose, experiments were performed using shake-flask cultures. Expression of only maltose phosphorylase (IMZ203) resulted in a growth rate of 0.09±0.03 h^{-1} on maltose. Co-expression of maltose phosphorylase and β-phosphoglucomutase (IMZ226) increased the aerobic growth rate to 0.21±0.01 h^{-1}, which is close to the growth rate of IMZ199 (*mal*Δ *mph*Δ *MAL11 MAL12*, 0.24±0.00 h^{-1}). The anaerobic growth rate on maltose increased from 0.03±0.01 h^{-1} (IMZ203) to 0.10±0.00 h^{-1} (IMZ226) (Table 3.4). These results showed that expression of both maltose phosphorylase and β-phosphoglucomutase is necessary for fast growth on maltose via the phosphorolytic pathway.

Anaerobic maltose catabolism via maltose phosphorylase increases the biomass yield on maltose

To analyze whether expression of the maltose phosphorylase-dependent pathway indeed led to increased free-energy (ATP) conservation, biomass yields on maltose were measured. Anaerobic maltose catabolism via the hydrolytic route (maltase) generates 3 ATP per maltose, whereas phosphorolytic maltose cleavage (maltose phosphorylase) should yield 4 ATP per maltose. This difference is mostly visible in the anaerobic biomass yield on maltose. Calculation of the theoretical biomass yields on maltose according to a model

presented previously [56] predicts that a catabolic route yielding 4 ATP should have a 33% higher biomass yield than a route yielding 3 ATP (Table 3.5). Biomass yields were experimentally determined in anaerobic maltose-limited chemostat cultures, as this is the most accurate method to determine growth stoichiometries. In view of the maximum specific growth rate of IMZ226 of 0.10 h^{-1} in anaerobic cultures (Table 3.4), experiments were performed at a dilution rate of 0.05 h^{-1}.

Table 3.5 Physiology of *Saccharomyces cerevisiae* strains IMZ199 (expressing maltase) and IMZ226 (expressing maltose phosphorylase and β-phosphoglucomutase) in anaerobic maltose-limited chemostat cultures at a dilution rate of 0.05 h^{-1}. Averages and mean deviations were obtained from duplicate experiments.

Strain	IMZ199	IMZ226
Relevant genotype	*mal*Δ *mph*Δ + *MAL11* + *MAL12*	*mal*Δ *mph*Δ + *MAL11* + *mapA* + *pgmB*
Dilution rate (h^{-1})	0.051±0.001	0.050±0.001
Theoretical biomass yield (g·gluc eq^{-1})[a]	0.066	0.088
Observed biomass yield (g·gluc eq^{-1})	0.067±0.000	0.085±0.000
$q_{maltose}$ (mmol·g $biomass^{-1}·h^{-1}$)	-2.11±0.04	-1.58±0.03
$q_{ethanol}$ (mmol·g $biomass^{-1}·h^{-1}$)	7.49±0.18	5.24±0.07
q_{CO2} (mmol·g $biomass^{-1}·h^{-1}$)	7.73±0.60	5.64±0.10
$q_{glycerol}$ (mmol·g $biomass^{-1}·h^{-1}$)	0.36±0.01	0.26±0.09
$q_{lactate}$ (mmol·g $biomass^{-1}·h^{-1}$)	0.04±0.00	0.03±0.00
$q_{pyruvate}$ (mmol·g $biomass^{-1}·h^{-1}$)	0.00±0.00	0.00±0.00
$q_{acetate}$(mmol·g $biomass^{-1}·h^{-1}$)	0.05±0.04	0.00±0.00
Residual maltose ($g·l^{-1}$)	0.24±0.00	0.69±0.23
Viability (%)	>99	>99
Carbon recovery (%)[b]	103±2	100±1

[a]The theoretical biomass yield was calculated for a growth rate of 0.05 h^{-1}, using a previously described model [56] and assuming a maintenance requirement of 1 mmol ATP·g $biomass^{-1}·h^{-1}$ [7].
[b]Calculations of the carbon recovery were based on a carbon content of biomass of 48% (w/w).

The maltose phosphorylase expressing strain IMZ226 showed a higher biomass yield on maltose than the maltase expressing strain IMZ199 (0.085±0.000 versus 0.067±0.000 g·g glucose $equivalents^{-1}$, Table 3.5). The difference (26%) was close to the values predicted by the model calculations (33%), albeit slightly lower. To validate the calculated yields, the anaerobic biomass yield on glucose was determined. Catabolism of glucose yields 2 ATP, which is per hexose (C_6) equivalent to catabolism of the disaccharide maltose (C_{12}) via the maltose phosphorylase route (4 ATP). The biomass yield of CEN.PK113-7D on glucose

under the same conditions was 0.088±0.001 g·glucose equivalent^{-1}, which is equal to the theoretical yield calculated (0.088 g·glucose equivalent^{-1}). These results indicated that anaerobic maltose catabolism in IMZ226 (*mal*Δ *mph*Δ *MAL11 mapA pgmB*) yields almost 4 ATP per maltose, compared to 3 ATP for IMZ199 (*MAL11 MAL12*).

Discussion

Critical steps in the implementation of a phosphorolytic pathway for disaccharide metabolism

This study provides a proof of principle for the functional replacement of a disaccharide hydrolase by a phosphorylase and for the associated improvement of free-energy (ATP) conservation. Using maltose metabolism in *S. cerevisiae* as the model, two critical steps were identified: complete elimination of maltose hydrolysis and co-expression of a phosphoglucomutase.

The biomass yield increase of the yeast strain expressing the bacterial maltose phosphorylase was slightly lower (26%) than theoretically expected (33%). Differences in strain backgrounds used for modelling (CBS8066) and experiments (CEN.PK) can have a small impact on the absolute yield values, but do not impact the relative difference in biomass yield between hydrolytic and phosphorolytic maltose cleavage within the same background. An alternative explanation could be that a fraction of the total maltose consumed in the chemostat experiments (1.58 mmol·g biomass^{-1}·h^{-1}) is cleaved via hydrolysis instead of phosphorolysis. An *in vivo* flux through maltase of 0.35 mmol·g biomass^{-1}·h^{-1} would result in the observed biomass yield increase of 26%. The observed *in vitro* maltase activity of 0.03 µmol·mg protein^{-1}·min^{-1} in the maltose knockout strain would, for a wide range of assumed soluble protein contents [40], be sufficient to explain the observed difference between the measured and predicted biomass yield. This underlines the need for complete removal of the hydrolytic activity to gain the maximum benefit from the phosphorolytic pathway. The remaining maltose hydrolase activity might originate from (an) unknown maltase(s) or from aspecific hydrolysis by enzymes that are involved in the hydrolysis of other sugars, such as isomaltose (Ima1-5p) [9, 48], sucrose (Suc2p) [11], glycogen (Gdb1p) [47], trehalose (Nth1p and Ath1p) [4, 29] or cell wall oligosaccharides (e.g. Cwh41p) [43]. A thorough functional analysis of

hydrolase-encoding genes in *S. cerevisiae* is therefore a prerequisite for further development of phosphorylase-based pathways.

In contrast to phosphorolytic cellobiose metabolism [44], efficient utilisation of maltose via maltose phosphorylase in *S. cerevisiae* required the co-expression of a β-phosphoglucomutase. The *S. cerevisiae* genome encodes for two phosphoglucomutases (*PGM1* and *PGM2*) [8]. Apparently, Pgm1p and Pgm2p can only efficiently convert α-glucose-1-phosphate into glucose-6-phosphate, not β-glucose-1-phosphate. Despite its undetectable β-phosphoglucomutase activity in cell extracts, IMZ203 (*mapA*) could still grow slowly on maltose. This can be explained by (i) a low *in vivo* activity that could not be determined *in vitro* due to suboptimal assay conditions or (ii) spontaneous mutarotation of β-glucose-1-phosphate into α-glucose-1-phosphate, which can be converted by an α-phosphoglucomutase into glucose-6-phosphate and enter glycolysis. Spontaneous mutarotation has previously been described for the conversion of β-glucose-6-phosphate into α-glucose-6-phosphate [5]. The requirement for a heterologous phosphoglucomutase activity will, in practice, depend on the sugar substrate. For example, sucrose, cellobiose and lactose phosphorolysis yields α-glucose-1-phosphate, which is a substrate for the native yeast Pgm1 and Pgm2 proteins, while maltose and trehalose phosphorolysis yields β-glucose-1-phosphate [3, 6, 14, 16].

Further engineering of free-energy (ATP) conservation from oligosaccharide metabolism

Under anaerobic conditions, the newly introduced maltose phosphorylase pathway yields 4 ATP instead of the 3 ATP that are formed during maltose fermentation via the native maltase-based pathway. This ATP yield is – per hexose – equal to the ATP yield on glucose (2 ATP per hexose). The ATP yield per hexose unit can be further increased by introducing a functional maltotriose phosphorylase and using maltotriose as substrate (Figure 3.2 and Table 3.6). Maltotriose can be imported by Mal11p via a maltotriose-proton-symport mechanism [25], which indirectly costs 1 ATP due to proton extrusion via the plasma-membrane H^+-ATPase [51, 56]. Maltotriose phosphorylase cleaves maltotriose into maltose and α-glucose-1-phosphate. Subsequent phosphorolysis of maltose followed by normal glycolysis will result in a net ATP yield of 2.33 ATP per hexose (Table 3.6). The only maltodextrin phosphorylase hitherto described that can use maltotriose as substrate for phosphorolysis originates

from the hyperthermophylic archaeon *Pyrococcus furiosus* [34]. We expressed the *PF1535* gene of *P. furiosus* in *S. cerevisiae*, but the activity at 30 °C was below the detection limit (<0.005 μmol·min^{-1}·mg protein^{-1}). Another option to increase free-energy conservation is the introduction of a maltose transporter with a uniporter mechanism (Figure 3.2 and Table 3.6). This will result in a net ATP yield of 5 ATP per maltose, equivalent to 2.5 ATP per hexose (Table 6). Recently, several single point mutations in the *MAL11* gene were reported to abolish proton coupling and to encode a maltose uniporter [49]. However, expression of these single mutant alleles (E120A, D123G and E167A) of *MAL11* in IMZ226 did not increase the biomass yield on maltose (data not shown), suggesting that these mutant alleles of *MAL11*, at least *in vivo*, still encode maltose-proton symporters.

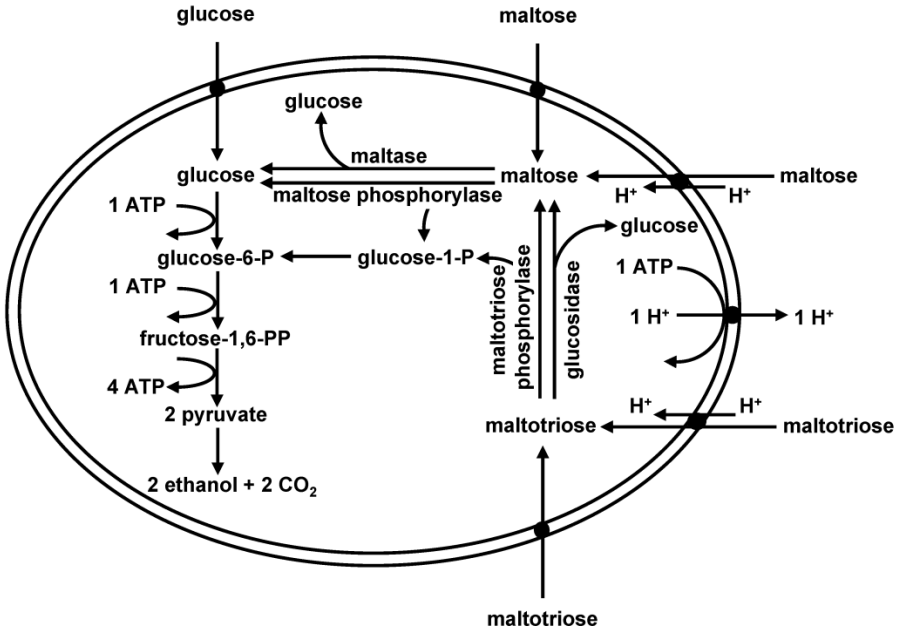

Figure 3.2 Schematic representation of different strategies to increase the net ATP yield of anaerobic maltose or maltotriose catabolism in *Saccharomyces cerevisiae* to above 2 ATP per hexose.

Table 3.6 Theoretical analysis of the net ATP yields of different catabolic pathways for maltose and maltotriose under anaerobic conditions, as depicted in Figure 3.2. Native *Saccharomyces cerevisiae* metabolism is indicated in grey.

Substrate	Transport mechanism	Cleavage mechanism	ATP/substrate	ATP/hexose
Glucose	Uniport	N.A.	2	2
Maltose	Proton symport	Hydrolysis	3	1.5
Maltose	Uniport	Hydrolysis	4	2
Maltose	Proton symport	Phosphorolysis	4	2
Maltose	Uniport	Phosphorolysis	5	2.5
Maltotriose	Proton symport	Hydrolysis	5	1.67
Maltotriose	Uniport	Hydrolysis	6	2
Maltotriose	Proton symport	Phosphorolysis	7	2.33
Maltotriose	Uniport	Phosphorolysis	8	2.67

Notwithstanding the current lack of a maltotriose phosphorylase with a high activity at 30 ˚C and/or a disaccharide uniporter, this study demonstrated the potential of increasing free-energy (ATP) conservation in *S. cerevisiae* on maltose and other disaccharides and identified key challenges. Ultimately, development of this concept may enable anaerobic production of compounds whose formation currently has a very low or zero ATP yield, such as the malate and lactate in engineered *S. cerevisiae* strains [1, 53, 58, 59].

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Chapter 4

In vivo analysis of the H^+/ATP stoichiometry of _Saccharomyces cerevisiae_ plasma-membrane ATPase Pma1p isoforms with increased _in vitro_ coupling ratios

Stefan de Kok, Duygu Yilmaz, Jean-Marc Daran, Jack T. Pronk and Antonius J.A. van Maris

Manuscript in preparation

Abstract

Plasma-membrane H^+-ATPase isoforms with increased H^+/ATP ratios represent a desirable asset in yeast metabolic engineering. *In vivo* proton coupling of two previously reported Pma1p isoforms (Ser800Ala, Glu803Gln) with increased *in vitro* H^+/ATP stoichiometries, was analyzed by measuring biomass yields of anaerobic maltose-limited chemostat cultures expressing only the different *PMA1* alleles. *In vivo* H^+/ATP stoichiometries of wild-type Pma1p and the two isoforms did not differ significantly.

Introduction

Plasma-membrane H$^+$-ATPases are ubiquitous enzymes that play an important role in eukaryotic physiology by using the free energy from ATP hydrolysis to pump protons from the cytosol, across the plasma membrane and out of the cell. In this way, cells maintain intracellular pH homeostasis and generate a proton-motive force (PMF), which can be used to drive many crucial transport processes [3, 17, 22]. *Saccharomyces cerevisiae* contains two distinct plasma-membrane H$^+$-ATPases encoded by the essential gene *PMA1* and the non-essential gene *PMA2* [15, 18]. The well-characterized plasma-membrane H$^+$-ATPase of *S. cerevisiae* [10, 11, 16] expels one proton per ATP molecule hydrolyzed [21, 24], even though the Gibbs free-energy of ATP hydrolysis (around –45 kJ·mol^{-1} under physiological conditions [4]) should be sufficient to drive export of 2 protons (+19 kJ·mol protons^{-1} at a PMF of –200 mV [17]). The H$^+$/ATP stoichiometry of the plasma-membrane H$^+$-ATPase determines the ATP requirement for cellular homeostasis and maintenance of the PMF. Moreover, it influences the biomass yield on substrates whose import makes use of the PMF (e.g. maltose and NH_4^+) [9, 21, 24], it can influence tolerance to both low pH and weak organic acids [1, 13, 23] and it can have a crucial impact on the stoichiometry and kinetics of organic acid production by engineered strains of *S. cerevisiae* [2, 14, 22]. Increasing the H$^+$/ATP stoichiometry of the *S. cerevisiae* plasma-membrane H$^+$-ATPase could therefore present many interesting opportunities for metabolic engineering.

Two isoforms of the *S. cerevisiae* plasma-membrane H$^+$-ATPase Pma1p have been described that displayed an increased *in vitro* H$^+$/ATP stoichiometry: Pma1p^{Ser800Ala} [8] and Pma1p^{Glu803Gln} [12]. However, no improved tolerance to low pH was observed after introducing the Glu803Gln mutation in *PMA1* [8]. The present study investigates whether the *in vivo* H$^+$/ATP stoichiometry of *S. cerevisiae* Pma1p isoforms can be analyzed via the anaerobic biomass yield on maltose of engineered strains. In *S. cerevisiae* maltose is imported via a proton-symport mechanism [21]. Due to subsequent proton export by the plasma-membrane H$^+$-ATPase at a stoichiometry of 1 H$^+$/ATP, conversion of the disaccharide maltose to ethanol only yields 3 ATP (1.5 ATP per hexose equivalent) [21, 24]. As a result, the anaerobic biomass yield on maltose is 25% lower per hexose equivalent than the anaerobic biomass yield on glucose (2 ATP per hexose) [21, 24]. In theory, an increased stoichiometry of the plasma-

membrane H^+-ATPase will increase the biomass yield on maltose due to a decreased energy-requirement of all processes that require proton extrusion (e.g. maintenance and generation of the PMF and import of maltose and NH_4^+). Even when only the lower ATP-requirement for maltose import is taken into account [24], a stoichiometry of 2 H^+/ATP is already expected to result in a 17% increase of the biomass yield on maltose. To characterize the *in vivo* H^+/ATP stoichiometry of the Ser800Ala and Glu803Gln isoforms of Pma1p, the wild-type *PMA1* allele was replaced by the corresponding *PMA1* mutant alleles in a *pma2*Δ background and the anaerobic biomass yields on maltose were compared to those of an isogenic *PMA1 pma2*Δ reference strain.

Materials and methods

To introduce the Ser800Ala (TCC→GCT) and Glu803Gln (GAA→CAA) mutations into *PMA1*, DNA from the *Kpn*I site in *PMA1* until the *Ngo*MIV site in *LEU1* was amplified from CEN.PK113-7D genomic DNA using primers PMA1 Fw and LEU1 Rv (for primers, see Table 4.1) and cloned into pENTR/D-TOPO using Gateway technology (Invitrogen, Carlsbad, USA), resulting in pUD109 (for plasmids, see Table 4.2). To introduce extra restriction sites in the intergenic region between *PMA1* and *LEU1,* DNA was amplified from pUD109 using primers LEU1p Fw and LEU1p Rv. The resulting PCR product was restricted with *Xba*I and *Hind*III and ligated into pUD109, resulting in pUD113. To introduce point mutations in *PMA1*, the *Kpn*I-*Sal*I fragment of pUD117 and pUD118, containing synthesized parts of *PMA1* including the Ser800Ala (TCC→GCT) and Glu803Gln (GAA→CAA) mutations, were ligated into pUD113, resulting in pUD119 and pUD120 (Table 4.2). To introduce the hygromycin B resistance marker *hphNT1*, a *Spe*I-*Bst*WI fragment of pUG-*hphNT1* was ligated into pUD119 and pUD120, resulting in pUD124 and pUD125 respectively. The *Kpn*I-*Ngo*MIV fragment of pUD124 and pUD125 was transformed to CEN.PK113-7D resulting in IMI058 and IMI059 respectively (for strains, see Table 4.3). Correct integration of the cassette was confirmed via PCR using primer pairs PMA1 Ctrl Fw/hphNT1 Rv and hphNT1 Fw/LEU1 Ctrl Rv. To remove the hygromycin B resistance marker gene *hphNT1*, IMI058 and IMI059 were transformed with pSH65 and – after marker removal via the Cre/loxP system [7] and curing of the pSH65 plasmid – designated IMI062 and IMI063, respectively. To knockout *PMA2*, a cassette

was amplified from pUG6 using primers PMA2 KO Fw and PMA2 KO Rv and transformed into CEN.PK113-7D, IMI062 and IMI063, resulting in IMK328, IMX051B and IMX052, respectively. Correct knockout was confirmed via PCR using the primer pairs PMA2 Ctrl Fw/KanMX Rv and KanMX Fw/PMA2 Ctrl Rv. Presence of the introduced point mutations was verified by duplicate amplification of *PMA1* using primers PMA1 Ctrl Fw and LEU1 Ctrl Rv and sequencing approximately 200 bp up- and downstream of the introduced mutations (Baseclear BV, Leiden, the Netherlands). Strain maintenance, yeast transformations and molecular biology techniques were performed as described previously [5].

Table 4.1 Primers used in this study.

Primer name	Sequence (5'→3')
PMA1 Fw	CACCGGGTACCAACATTTACAACGCTG
LEU1 Rv	CAACTCTTCTGACCTTTCTGCC
LEU1p Fw	CTCTAGACACTAGTATGCCGTACGTGACTCAGTTTAGTCTGACCTTC
LEU1p Rv	CCTTCGAAAGCTTGTGGAG
PMA1 Ctrl Fw	GGATCCACCAAGAGACGATACTG
LEU1 Ctrl Rv	CCGAAATATGGAACGCCGAACTG
hphNT1 Fw	ACGCGGATTTCGGCTCCAAC
hphNT1 Rv	AGACGTCGCGGTGAGTTCAG
PMA2 KO Fw	TCGTTGCTGTGTGCTAGTACAATTTAAGCAAAAGGAAACTGTTTTGC GTTCAGCTGAAGCTTCGTACGC
PMA2 KO Rv	CTTGGTATCGACAAATTGAAATGAAAATGAGGAATAACAAAAAGGA GATCGCATAGGCCACTAGTGGATCTG
PMA2 Ctrl Fw	GGCGGTGTGATGGTACTTC
PMA2 Ctrl Rv	CGGCCTACTTCTGATATGTGG
KanMX Fw	TCGTATGTGAATGCTGGTCG
KanMX Rv	CGCACGTCAAGACTGTCAAG

Table 4.2 Plasmids used in this study.

Plasmid	Characteristic	Reference/source
pENTR/D-TOPO	Gateway entry clone	Invitrogen, USA
pUG6	PCR template for *loxP-KanMX4-loxP* cassette	[7]
pSH65	Centromeric plasmid, *ble*, P_{GAL1}-*Cre*-T_{CYC1}	[7]
pUG-*hphNT1*	PCR template for *loxP-hphNT1-loxP* cassette	[5]
pUD117	pUC57, synthetic construct *`PMA1$^{S800A}$`*	Baseclear BV, the Netherlands
pUD118	pUC57, synthetic construct *`PMA1$^{E803Q}$`*	Baseclear BV, the Netherlands
pUD109	Gateway entry clone, *`PMA1-LEU1`*	This study
pUD113	Gateway entry clone, *`PMA1-multiple cloning site-LEU1`*	This study
pUD119	Gateway entry clone, *`PMA1$^{S800A}$-multiple cloning site-LEU1`*	This study
pUD120	Gateway entry clone, *`PMA1$^{E803Q}$-multiple cloning site-LEU1`*	This study
pUD124	Gateway entry clone, *`PMA1$^{S800A}$-loxP-hphNT1-loxP-LEU1`*	This study
pUD125	Gateway entry clone, *`PMA1$^{E803Q}$-loxP-hphNT1-loxP-LEU1`*	This study

Table 4.3 *Saccharomyces cerevisiae* strains used in this study.

Strain	Relevant genotype	Reference
CEN.PK113-7D	MATa *PMA1 PMA2*	[6, 20]
IMK328	MATa *PMA1 pma2::loxP-KanMX4-loxP*	This study
IMI058	MATa *PMA1^{S800A}-loxP-hphNT1-loxP PMA2*	This study
IMI059	MATa *PMA1^{E803Q}-loxP-hphNT1-loxP PMA2*	This study
IMI062	MATa *PMA1^{S800A} PMA2*	This study
IMI063	MATa *PMA ^{E803Q} PMA2*	This study
IMX051B	MATa *PMA1^{S800A} pma2::loxP-KanMX4-loxP*	This study
IMX052	MATa *PMA1^{E803Q} pma2::loxP-KanMX4-loxP*	This study

Results and discussion

To analyze the *in vivo* H^+/ATP stoichiometry of the Pma1p^{Ser800Ala} and Pma1p^{Glu803Gln} isoforms, anaerobic chemostat experiments with maltose as the sole carbon source were performed at pH 5.0 as described previously [5]. To prevent evolutionary adaptation, the cultures were sampled within 12 volume changes. In agreement with model predictions based on a H^+/ATP stoichiometry of 1.0 and previous observations [24], the anaerobic biomass yield on maltose of the reference strain CEN.PK113-7D (*PMA1 PMA2*) was 24±0% lower per hexose equivalent than the anaerobic biomass yield on glucose (Table 4.4). The biomass yield of the engineered strains IMX051B (*PMA1^{Ser800Ala} pma2*Δ) and IMX052 (*PMA1^{Glu803Gln} pma2*Δ) was not higher than the yield of the

reference strain CEN.PK113-7D (*PMA1 PMA2*) or the isogenic strain IMK328 (*PMA1 pma2*Δ) (Table 4.4). At the end of the chemostat experiments, genomic DNA was extracted and used for duplicate amplification of part of *PMA1*. Subsequent sequencing confirmed that the introduced mutations were still present. Apparently, the Ser800Ala and Glu803Gln mutations in *PMA1* did not increase the *in vivo* H^+/ATP stoichiometry under the tested conditions, in contrast to what has been reported previously using *in vitro* assays [8, 12]. To test whether these contradictory results were due to differences in pH used in the *in vivo* (pH 5.0) and *in vitro* (pH 6.7) experiments, the chemostat experiments were repeated at pH 6.7. Also under these conditions, the difference in anaerobic biomass yield on glucose and maltose of the reference strain CEN.PK113-7D (*PMA1 PMA2*) at pH 6.7 was 24±0% (Table 4.4). Interestingly, at pH 6.7 deletion of *PMA2* seemed to increase the biomass yield on maltose by 5.4±0.0% when comparing the reference strain CEN.PK113-7D (*PMA1 PMA2*) and IMK328 (*PMA1 pma2*Δ). However, the biomass yields on maltose of the engineered strains IMX051B (*PMA1*^{Ser800Ala} *pma2*Δ) and IMX052 (*PMA1*^{Glu803Gln} *pma2*Δ) were identical to the isogenic reference strain IMK328 (*PMA1 pma2*Δ) (Table 4.4). Thus, at both pH 5.0 and pH 6.7 introduction of the Ser800Ala and Glu803Gln isoforms in Pma1p did not increase the *in vivo* H^+/ATP stoichiometry.

Table 4.4 Anaerobic biomass yields of *Saccharomyces cerevisiae* strains CEN.PK113-7D (*PMA1 PMA2*), IMK328 (*PMA1 pma2*Δ), IMX051B (*PMA1*^{Ser800Ala} *pma2*Δ) and IMX052 (*PMA1*^{Glu803Gln} *pma2*Δ) in anaerobic sugar-limited chemostat cultures at a dilution rate of 0.10 h^{-1}. Averages and mean deviations were obtained from duplicate cultures.

			Biomass yield (g·g gluc eq^{-1})	
Strain	Relevant genotype	Carbon source	pH 5.0	pH 6.7
CEN.PK113-7D	*PMA1 PMA2*	Glucose	0.095±0.002	0.087±0.001
CEN.PK113-7D	*PMA1 PMA2*	Maltose	0.072±0.000	0.066±0.000
IMK328	*PMA1 pma2*Δ	Maltose	0.072±0.001	0.070±0.000
IMX051B	*PMA1*^{Ser800Ala} *pma2*Δ	Maltose	0.073±0.001	0.069±0.002
IMX052	*PMA1*^{Glu803Gln} *pma2*Δ	Maltose	0.073±0.001	0.069±0.001

In vitro studies are an essential tool in gaining increased understanding of membrane proteins such as H^+-ATPase [10, 11, 16]. Several factors may explain why the Ser800Ala and Glu803Gln isoforms of Pma1p, which were clearly shown to translocate 2-3 protons per ATP *in vitro* [8, 12], did not lead to a significantly increased *in vivo* H^+/ATP stoichiometry in the anaerobic maltose-limited cultures. Even when *in vitro* studies attempt to mimic *in vivo* conditions (e.g. pH and osmolarity), subtle differences in membrane composition between the plasma membrane and secretory vesicle membrane [19] might affect the three-dimensional structure and functioning of the plasma-membrane H^+-ATPase. Additionally, thermodynamics of the proton-motive force (PMF) and/or ATP hydrolysis may be different under *in vitro* and *in vivo* conditions. If the PMF in the secretory vesicles, which has not been measured [8, 12], is significantly lower than the *in vivo* PMF, this would make an increased H^+/ATP stoichiometry thermodynamically easier to achieve *in vitro*, but not *in vivo*. This difference between the *in vitro* and *in vivo* thermodynamic potential of the plasma-membrane H^+-ATPase becomes even more striking for the free energy of ATP hydrolysis. In the *in vitro* assays, ADP and inorganic phosphate were not added to the reaction mixture and only ATP was added from the start. Especially during the early stages of the assay, which coincides with the determination of the H^+/ATP stoichiometry, this created a non-physiologically high driving force for ATP hydrolysis, which will drastically exceed the estimated –45 $kJ \cdot mol^{-1}$ under physiological conditions [4]. The method presented in this study, in which *in vivo* proton coupling of plasma-membrane H^+-ATPase isoforms was analyzed via its impact on the biomass yields of anaerobic maltose-grown cultures, provides a useful tool in the continuing search for Pma1p isoforms and/or heterologous plasma-membrane H^+-ATPases with an *in vivo* H^+/ATP ratio above 1.0 in growing yeast cultures.

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Chapter 5

Engineering topology and kinetics of sucrose metabolism in *Saccharomyces cerevisiae* for improved ethanol yield

Thiago O. Basso[*], Stefan de Kok[*], Marcelo Dario, Júlio Cézar A. do Espirito-Santo, Gabriela Müller, Paulo S. Schlölg, Carlos P. Silva, Aldo Tonso, Jean-Marc Daran, Andreas K. Gombert, Antonius J.A. van Maris, Jack T. Pronk and Boris U. Stambuk

*These authors contributed equally to this publication

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Abstract

Sucrose is a major carbon source for industrial bioethanol production by *Saccharomyces cerevisiae*. In yeasts, two modes of sucrose metabolism occur: (i) extracellular hydrolysis by invertase, followed by uptake and metabolism of glucose and fructose, and (ii) uptake via sucrose-proton symport followed by intracellular hydrolysis and metabolism. Although alternative start codons in the *SUC2* gene enable synthesis of extracellular and intracellular invertase isoforms, sucrose hydrolysis in *S. cerevisiae* predominantly occurs extracellularly. In anaerobic cultures, intracellular hydrolysis theoretically enables a 9% higher ethanol yield than extracellular hydrolysis, due to energy costs of sucrose-proton symport. This prediction was tested by engineering the promoter and 5' coding sequences of *SUC2*, resulting in predominant (94%) cytosolic localization of invertase. In anaerobic sucrose-limited chemostats, this i*SUC2*-strain showed an only 4% increased ethanol yield and high residual sucrose concentrations indicated suboptimal sucrose-transport kinetics. To improve sucrose-uptake affinity, the i*SUC2*-strain was subjected to 90 generations of laboratory evolution in anaerobic sucrose-limited chemostat cultivation, resulting in a 20-fold decrease of residual sucrose concentrations and a 10-fold increase of the sucrose-transport capacity. A single-cell isolate showed an 11% higher ethanol yield on sucrose in chemostat cultures than an isogenic *SUC2* reference strain, while transcriptome analysis revealed elevated expression of *AGT1*, encoding a disaccharide-proton symporter, and other maltose-related genes. After deletion of both copies of the duplicated *AGT1*, growth characteristics reverted to that of the unevolved *SUC2* and i*SUC2* strains. This study demonstrates that engineering the topology of sucrose metabolism is an attractive strategy to improve ethanol yields in industrial processes.

Introduction

Mainly used as an automotive fuel, bioethanol is the single largest product of industrial biotechnology by volume, with an estimated global production of $87 \cdot 10^9$ litres in 2010 [44]. Currently, the predominant feedstocks for bioethanol production are corn starch or sugar-cane sucrose. The sugars derived from these agricultural crops are fermented under anaerobic conditions by the yeast *Saccharomyces cerevisiae*. While a huge research effort is underway to unlock additional, lignocellulosic biomass feedstocks [62], improvement of sugar-cane sucrose-based ethanol production needs to continue due to the important role of feedstock costs in the overall process economics (up to 70% of the ethanol production cost [38]). Given the large volumes of industrial ethanol production, even small increases in the ethanol yield on sugar are economically significant.

S. cerevisiae can metabolize sucrose, the major sugar in cane juice and in molasses, in two ways. In the first and predominant mechanism, sucrose is hydrolyzed by an extracellular invertase encoded by the *SUC2* gene. Hydrolysis yields glucose and fructose, which enter into the cell by facilitated diffusion via hexose transporters encoded by members of the *HXT* gene family [36]. In the second mechanism, sucrose can be actively transported into the cells by a proton-symport mechanism and hydrolyzed intracellularly [5, 46, 51]. Both extra- and intracellular invertase are encoded by the same gene (*SUC2*), which has two different start codons, leading to synthesis of active invertase isoforms of 532 and 512 amino acids, respectively [13, 14, 24, 55]. The larger and predominant Suc2p isoform, which is glycosylated, is exported across the plasma membrane, partially retained in the cell wall and partially released in the extracellular medium. The shorter and non-glycosylated isoform of invertase is retained in the cytosol where it, together with intracellular maltase (encoded by the *MALx2* genes), contributes to the intracellular sucrose-hydrolyzing capacity of *S. cerevisiae* [3, 13, 23]. Already in 1982, it was shown that wild-type *S. cerevisiae* has a low capacity for sucrose uptake via a sucrose-proton symporter [46], an activity that was later attributed to the *AGT1* (*MAL11*) gene product [51]. However, in sucrose-grown cultures of wild-type *S. cerevisiae* strains, sucrose hydrolysis occurs almost exclusively extracellularly [5, 11, 13, 23, 53].

Anaerobic fermentation of glucose or fructose via the yeast glycolytic pathway yields 2 mol ATP per mol of hexose. Therefore, 4 mol ATP are formed per mol of sucrose when this disaccharide is fermented via the extracellular

hydrolysis pathway. When sucrose is metabolized via the intracellular hydrolysis pathway, the single proton that is taken up along with sucrose [46, 51] has to be expelled by the plasma-membrane H^+-ATPase (Pma1p) to maintain the proton-motive force across this membrane and to prevent intracellular acidification. In *S. cerevisiae* and *Neurospora crassa*, the stoichiometry of the plasma-membrane H^+-ATPase is 1 H^+ per ATP [41, 47, 60, 63]. This reduces the net ATP yield of anaerobic sucrose fermentation via the intracellular hydrolysis pathway to only 3 ATP per mol sucrose.

During fermentative growth on sugars, part of the carbon source is used for the production of yeast biomass and glycerol, which is needed to reoxidize the 'excess' NADH formed in biosynthetic reactions [59]. Biomass and glycerol production from glucose/fructose require a net input of free energy (ATP), which is provided by alcoholic fermentation of the remainder of the feedstock. Therefore, in growing cultures, a decrease in the ATP yield from alcoholic fermentation will result in a larger fraction of the sugar being converted to ethanol, with a concomitant lower biomass yield. Consequently, sucrose fermentation via the intracellular pathway (3 mol ATP per mol sucrose) should enable a higher ethanol yield on sucrose than its fermentation via the extracellular pathway (4 ATP per sucrose).

Weusthuis *et al.* (1993) studied the impact of disaccharide-proton symport on biomass and product yields by comparing anaerobic growth of *S. cerevisiae* on maltose (which is transported by a maltose-proton symporter and intracellularly hydrolyzed by maltase) and glucose. They demonstrated that, consistent with model predictions, biomass and ethanol yields in anaerobic maltose-limited cultures were 25% lower and 8% higher, respectively, than in glucose-limited cultures. Similar yield differences are theoretically expected between anaerobic sucrose-limited cultures of *S. cerevisiae* utilizing sucrose via the intracellular pathway and via the extracellular pathway.

The goal of the present study was to investigate whether a relocation of sucrose hydrolysis from the extracellular space to the cytosol can be used to improve ethanol yields on sucrose and which additional steps may be required to improve sucrose utilization by strains that only express intracellular invertase. Growth and product formation by a strain with a modified *SUC2* gene were compared with that of the parental strain in anaerobic sucrose-limited chemostat cultures. Subsequently, evolutionary engineering was used to improve sucrose-uptake kinetics and an evolved strain was characterized for growth and product

formation in chemostat cultures. Transcriptome analysis and gene deletion studies were used to identify genetic changes in the evolved strain that contribute to its improved sucrose-uptake kinetics.

Materials and methods

Yeast strains and maintenance

The *S. cerevisiae* strains used in this study (Table 5.1) are congenic members of the CEN.PK family [20, 58]. Stock cultures were grown in shake flasks containing synthetic medium [61] with 20 $g \cdot l^{-1}$ glucose as carbon source. After overnight growth, 20% (v/v) glycerol was added and 1-ml aliquots were stored at –80 °C.

Table 5.1 *Saccharomyces cerevisiae* strains used in this study.

Strain	Relevant genotype	Source
CEN.PK113-7D	MATa *URA3 TRP1 SUC2*	P. Kötter, Germany
BY4741 *agt1*Δ	MATa *agt1::loxP-KanMX4-loxP*	Euroscarf, Germany
BSY021-34B	MATa *ura3-52 trp1-289 TRP1*-P_{ADH1}::i*SUC2*	This study
IMI056	MATa *URA3 trp1-289 TRP1*-P_{ADH1}::i*SUC2*	This study
IMM007	IMI056 evolved	This study
IMM008	IMI056 evolved *agt1::loxP-KanMX4-loxP*	This study
IMM009	IMI056 evolved *agt1::loxP-KanMX4-loxP* *agt1::loxP-hph*NT1*-loxP*	This study

Strain construction

All transformations were carried out as described previously [25]. Primers iSUC2 Repl Fw and iSUC2 Repl Rv (Table 5.2) were used to amplify a tranformation cassette from plasmid pFA6a-*TRP1*-P_{ADH1} [18], carrying the *TRP1* marker gene and the *ADH1* promoter. The resulting transformation cassette contained homology to regions immediately up- and downstream of the second start codon of the *SUC2* gene after 60 bp, thereby removing 20 N-terminal amino acids of the resulting protein [13, 14]. Transformation and selection for tryptophan prototrophy resulted in strain BSY021-34B. Correct integration was verified via diagnostic PCR with primers iSUC2 Ctrl Fw and iSUC2 Ctrl Rv (Table 5.2). To restore the uracil auxotrophy that could interfere with chemostat characterization [4], the 523 bp *Nde*I/*Stu*I fragment from pRS406 [48] was used to transform strain BSY021-34B, resulting in IMI056. For deletion of *AGT1* in IMM007, a knockout cassette with the *KanMX4* marker

was amplified from BY4741 *agt1*Δ genomic DNA using primers AGT1 KO Fw A and AGT1 KO Rv A (Table 5.2). Transformation and selection on agar plates containing 200 mg·l^{-1} G418 (Invivogen, San Diego, USA) resulted in IMM008. Correct integration of the knockout cassette was verified via diagnostic PCR with primer pairs AGT1 Ctrl Fw/KanMX4 Ctrl Rv and KanMX4 Ctrl Fw/AGT1 Ctrl Rv (Table 5.2). For deletion of the second copy of *AGT1* in IMM008, a knockout cassette with the *hphNT1* marker was amplified from pUG-*hphNT1* [16] using primers AGT1 KO Fw B and AGT1 KO Rv B (Table 5.2). Transformation and selection on agar plates containing 200 mg·l^{-1} hygromycin B (Invivogen, San Diego, USA) resulted in IMM009. Correct integration of the knockout cassette was verified via diagnostic PCR with primer pairs AGT1 Ctrl Fw/hphNT1 Rv and hphNT1 Fw/AGT1 Ctrl Rv (Table 5.2). Molecular biology techniques were performed as described previously [16].

Table 5.2 Primers used in this study

Primer name	Sequence (5'→3')
iSUC2 Repl Fw	TTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCATCAGA ATTCGAGCTCGTTTAAAC
iSUC2 Repl Rv	GTGTGAAGTGGACCAAAGGTCTATCGCTAGTTTCGTTTGTC ATTGTATATGAGATAGTTG
iSUC2 Ctrl Fw	CTCCCCCGTTGTTGTCTCAC
iSUC2 Ctrl Rv	GGCACTGTACTCCCAGTT
AGT1 KO Fw A	GCGAGTTGCAAGAATCTCTACG
AGT1 KO Rv A	GATGACGACCACATGGGTTTG
AGT1 Ko Fw B	TCATTTCATTGGTAAGCAAGAAGAAGGCTGCCTCAAAAAAT GAGGATAAAAACATCAGCTGAAGCTTCGTACGC
AGT1 KO Rv B	CATTTATCAGCTGCATTTAATTCTCGCTGTTTTATGCTTGAG GACTGACTGATACGCATAGGCCACTAGTGGATCTG
AGT1 Ctrl Fw	GCCTCTTTCCACCACTTTG
AGT1 Ctrl Rv	ACGAGGACTGTCAGACCATTG

Medium and cultivation

Precultures for chemostat experiments were grown until the mid-exponential growth phase at 30 °C in 500-ml shake flasks containing 100 ml synthetic medium [61] with 20 g·l^{-1} sucrose in an Innova incubator shaker (New Brunswick Scientific, Edison, USA). A synthetic medium with 25 g·l^{-1} sucrose was used for all chemostat experiments [61]. Sucrose was filter-sterilized as a 50% (w/v) solution before being transferred to the medium. The medium was supplemented with the anaerobic growth factors ergosterol (10 mg·l^{-1}) and

Tween80 (420 mg·l^{-1}) dissolved in ethanol. Antifoam Emulsion C (Sigma, St. Louis, USA) was autoclaved separately (121 ˚C, 20 min) as a 20% (w/v) solution and added to a final concentration of 0.2 g·l^{-1}. Anaerobic chemostat fermentations were run in 2 litre laboratory bioreactors (Applikon, Schiedam, the Netherlands) at 800 rpm and 30 ˚C. The working volume was kept at 1.0 litre using an effluent pump, controlled by an electric level sensor. The exact working volume was measured at the end of each experiment. The pH was kept at 5.0 via automatic addition of 2 M KOH. Cultures were sparged with 500 ml·min^{-1} nitrogen (<10 ppm oxygen). To minimize oxygen diffusion into the system, bioreactors were equipped with Norprene tubing and Viton O-rings and the medium vessels were flushed with nitrogen gas. Chemostat cultivations were preceded by batch cultivations under the same conditions. After sucrose exhaustion, which was indicated by a rapid decrease in CO_2 production, the cultivations were switched to continuous mode at a dilution rate of 0.10 h^{-1}. Culture purity was routinely monitored by phase contrast microscopy.

Analytical methods

Cultures were assumed to be in steady state when, after at least five volume changes, the culture dry weight and the specific carbon dioxide production rate varied less than 2% over 2 volume changes. Culture dry weights were determined via filtration of 20-ml samples over dry preweighed nitrocellulose filters (Gelman laboratory, Ann Arbor, USA) with a pore size of 0.45 µm. After removal of the medium, the filters were washed twice with demineralized water, dried in a microwave oven for 20 min at 360 W and weighed. Supernatants were obtained after centrifugation of culture broth. Residual sugars were sampled as described previously [39]. Supernatants and media were analyzed via HPLC using an Aminex HPX-87H ion exchange column (BioRad, Richmond, USA) at 60 ˚C with 5 mM H_2SO_4 as the mobile phase at 0.6 ml·min^{-1}. Ethanol, glycerol, succinate and lactate were detected by a Waters 2410 refraction index detector. Pyruvate and acetate were detected by a Waters 2487 UV detector at 214 nm. Ethanol concentrations were corrected for evaporation as described previously [28]. Sucrose, glucose and fructose concentrations were analyzed via an enzymatic assay (10716260035, Boehringer Mannheim, Mannheim, Germany) according to manufacturer's instructions. Exhaust gas from the bioreactor was cooled in a condenser (2 ˚C) to minimize ethanol evaporation and dried in a Perma Pure Dryer (Permapure, Toms River, USA). CO_2 concentrations in the

off-gas were analyzed with a Rosemount NGA 200000 gas analyzer (Rosemount Analytical, Orrvile, USA).

Invertase assay

For invertase activity measurements, cells were collected from chemostats, washed twice and resuspended in cold water. Extracellular invertase activity was determined by measuring glucose formation at 30 °C in 50 mM Tris-succinate buffer (pH 5.0) with 150 mM sucrose using cells pre-incubated with 50 mM sodium fluoride [49]. To measure total invertase activity, cells were permeabilized as described previously [50] with ethanol, 10% (v/v) Triton X-100 and toluene (1:4:1), washed twice and then incubated at 30 °C with 100 mM sucrose in 50 mM Tris-succinate buffer (pH 5.0). Glucose formation was analyzed via an enzymatic assay as described above. All assays were carried out in triplicate with a standard deviation of <10%.

Sucrose-proton symport assay

Proton symport during sucrose uptake was determined by recording pH changes in yeast suspensions [51]. Yeast cells were suspended to a cell density of 25 $g \cdot l^{-1}$ in 25 mM K-phtalate buffer (pH 5.0) and placed in a water-jacketed vessel in a total volume of 5 ml. The suspension was mixed with a magnetic stirrer at 30 °C. Changes of pH triggered by the addition of sucrose (70 mM final concentration) were monitored with a pH sensor. To calculate the rate of proton uptake, a calibration curve was obtained by the addition of 100 to 500 nmol of NaOH to the cell suspension. Initial rates of sucrose-induced proton uptake were calculated from the slope of the first 10-20 s after sucrose addition, subtracting the basal rate of proton uptake observed before addition of sucrose. All assays were carried out at least in triplicate with standard deviations of <15%.

Microarray processing and analysis

DNA microarray analyses were performed with the S98 Yeast GeneChip arrays (Affymetrix, Santa Clara, USA) as previously described [15, 42]. Cells were transferred directly from chemostats into liquid nitrogen and processed according to manufacturer's instructions (Affymetrix, Santa Clara, USA) with the following modifications: double-stranded cDNA synthesis was carried out using 15 µg total RNA and the components of the One Cycle cDNA Synthesis

Kit (Affymetrix, Santa Clara, USA). Double-stranded cDNA was purified using the GeneChip Sample Cleanup Module (Qiagen, Hilden, Germany) before *in vitro* transcription and labeling with the GeneChip IVT Labelling Kit (Affymetrix, Santa Clara, USA). Finally, labelled cRNA was purified using the GeneChip Sample Cleanup Module prior to fragmentation and hybridization of 15 µg biotinylated cRNA [17]. Acquisition and quantification of array images and data filtering were performed using Affymetrix GeneChip Operating Software, version 1.2. The Significance Analysis of Microarrays (SAM, version 2.23A) [57] add-in to Microsoft Excel was used for comparison of replicate array experiments of IMI056 and IMM007 under sucrose limitation conditions. Transcript data have been deposited in the Genome Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE30535. Groups of corresponsive genes were consulted for enrichment of functional annotation according to Gene Ontology [2] as previously described [34].

Results

Topology of sucrose metabolism: theoretical analysis of impacts on biomass and ethanol yield

A theoretical analysis of the impact of the envisaged metabolic engineering strategy was based on the assumption that biomass composition, ATP requirement for maintenance and assimilatory pathways in *S. cerevisiae* are independent of the topology of sucrose metabolism. As discussed above, the energy requirement for sucrose transport limits the net ATP yield from anaerobic sucrose metabolism via intracellular hydrolysis to 1.5 ATP per hexose equivalent, as compared to 2 ATP per hexose equivalent for glucose metabolism and for sucrose metabolism via extracellular hydrolysis. As a consequence, compared to a scenario where sucrose hydrolysis occurs extracellularly, one additional sucrose molecule must be fermented to ethanol for every three sucrose molecules entering the cell, leading to a 25% lower biomass yield on sucrose. The same difference is expected for the glycerol yield since, in the absence of osmotic stress, glycerol production by anaerobic *S. cerevisiae* cultures is strictly coupled to growth [59].

The additional sucrose consumed to energize sucrose-proton symport is completely converted to ethanol and carbon dioxide and not to biomass or

glycerol. Therefore, the ethanol yield on sucrose in cells using the intracellular pathway should be higher than in cells using the extracellular pathway. The increase in ethanol yield of a strain that exclusively employs the intracellular hydrolysis pathway relative to a strain using the extracellular pathway can be predicted according to Equation 5.1:

$$Y_{SucE,intra} = 0.75\ Y_{SucE,extra} + 0.25\ Y_{SucE,max} \quad (5.1)$$

In Equation 5.1, $Y_{SucE,intra}$ and $Y_{SucE,extra}$ (mol ethanol·mol sucrose^{-1}) are the ethanol yields on sucrose of strains that exclusively hydrolyze sucrose intracellularly and extracellularly, respectively. $Y_{SucE,max}$ is the maximum ethanol yield obtained upon complete dissimilation of sucrose via alcoholic fermentation. After substitution of 4 mol ethanol·mol sucrose^{-1} for $Y_{SucE,max}$, the relative ethanol yield of a strain using the intracellular sucrose hydrolysis pathway ($R = Y_{SucE,intra}/Y_{SucE,extra}$) is described by Equation 5.2:

$$R = 0.75 + 1/Y_{SucE,extra} \quad (5.2)$$

In anaerobic sucrose-limited chemostat cultures (D=0.10 h^{-1}) of the reference strain *S. cerevisiae* CEN.PK113-7D, the ethanol yield on sucrose was 2.96 mol·mol^{-1} (Table 5.3). Assuming that the contribution of intracellular sucrose hydrolysis in this strain is negligible [13, 23] and that, therefore, this ethanol yield corresponds to $Y_{sucE,extra}$, this predicts that a switch to intracellular sucrose hydrolysis enables an 8.7% increase of the ethanol yield on sucrose (Equation 5.2). This increase is virtually the same as the 8% difference in ethanol yields between anaerobic chemostat cultures grown on maltose (which is taken up by proton symport and hydrolyzed intracellularly) and on glucose (transported by facilitated diffusion), as predicted and demonstrated by Weusthuis *et al.* (1993).

Engineering of the *SUC2* gene results in relocation of invertase to the cytosol

To verify the predicted effect of pathway topology on biomass and ethanol yields in sucrose-grown cultures, the localization of invertase in *S. cerevisiae* was changed by genetic modification. The *S. cerevisiae SUC2* gene encodes both the intracellular non-glycosylated invertase and the secreted glycosylated form of the enzyme [13, 14]. To constitutively and exclusively express the intracellular form of invertase, the strong, constitutive *ADH1* promoter was used to replace the native, inducible *SUC2* promoter. Moreover, the transformation cassette containing the *ADH1* promoter was designed such that its integration removed the first 60 nucleotides of the *SUC2* gene, which encode the 20-amino-acid N-terminal signal peptide for extracellular targeting [14].

Localization of invertase in the engineered 'i*SUC2*' strain IMI056 and in the reference strain CEN.PK113-7D (*SUC2*) was analyzed by comparing total and extracellular sucrose-hydrolyzing activity. In the reference strain, 90% of the total invertase activity was found extracellularly (Table 5.3). In the i*SUC2* strain IMI056, 94% of the total invertase activity was found intracellularly (Table 5.3). The total invertase activity in the i*SUC2* strain was higher than in the reference strain, probably as a result of the use of the strong *ADH1* promoter used for expression of the engineered i*SUC2* gene. The low activity recovered in the extracellular fraction of the i*SUC2* strain IMI056 (Table 5.3) may be due to release of some intracellular invertase (e.g. by lysis) or a low activity of another, unknown extracellular sucrose-hydrolyzing enzyme. Since analysis of invertase activity was based on measurement of glucose release, this activity might also reflect release from the cytosol via Hxt transporters of some glucose originating from intracellular sucrose hydrolysis [8, 31].

Cytosolic expression of invertase has a minor impact on growth stoichiometry

To analyze the impact of retargeting invertase to the cytosol on biomass and product formation, growth of the i*SUC2* strain IMI056 was studied in anaerobic sucrose-limited chemostat cultures ($D=0.10\ h^{-1}$) and compared to that of the isogenic reference strain CEN.PK113-7D (*SUC2*).

Biomass and ethanol yields on sucrose monohydrate (0.094 g biomass·g sucrose·H_2O^{-1} and 0.38 g ethanol·g sucrose·H_2O^{-1}, respectively; Table 5.3) of the *SUC2*-reference strain were comparable to previously published data on growth of this strain in anaerobic glucose-limited chemostats (0.09 g biomass·g $glucose^{-1}$ and 0.40 g ethanol·g $glucose^{-1}$, respectively [1]). The sucrose-proton symport capacity assayed in samples from chemostat cultures of the reference strain was 7-fold lower than the sucrose-consumption rate in these cultures (Table 5.3). Proton-symport activities were assayed at a near-saturating sucrose concentration of 70 mM. Given the estimated K_m of high-affinity sucrose-proton symport in *S. cerevisiae* of 5-8 mM [51], *in situ* transport activities in the chemostat cultures, in which the residual sucrose concentration was below 0.3 mM (Table 5.3), are expected to be at least an order of magnitude lower. These observations are consistent with a predominantly extracellular hydrolysis of sucrose in the *SUC2* reference strain CEN.PK113-7D.

Table 5.3 Physiology of *S. cerevisiae* strains CEN.PK113-7D (*SUC2*), IMI056 (i*SUC2*), IMM007 (i*SUC2* evolved) and IMM009 (i*SUC2* evolved *agt1*Δ/*agt1*Δ) in anaerobic sucrose-limited chemostat cultures at a dilution rate of 0.10 h^{-1}. Averages and mean deviations were obtained from independent duplicate experiments.

Strain	CEN.PK113-7D	IMI056	IMM007	IMM009
Relevant genotype	*SUC2*	i*SUC2*	i*SUC2* evolved	i*SUC2* evolved *agt1*Δ/*agt1*Δ
Actual dilution rate (h^{-1})	0.103±0.001	0.101±0.001	0.101±0.000	0.102±0.001
Sucrose consumption rate (mmol·g $DW^{-1}·h^{-1}$)	3.11±0.03	3.15±0.02	4.25±0.05	2.92±0.02
Biomass yield (g·mol $sucrose^{-1}$)	33.9±0.4	31.7±0.4	23.8±0.7	34.2±0.4
Relative biomass yield[a]	1.00	0.94±0.02	0.70±0.02	1.01±0.02
Ethanol yield (mol·mol $sucrose^{-1}$)	2.96±0.01	3.09±0.06	3.30±0.06	2.97±0.02
Relative ethanol yield[a]	1.00	1.04±0.02	1.11±0.02	1.01±0.01
Glycerol yield (mol·mol $sucrose^{-1}$)	0.25±0.00	0.22±0.00	0.20±0.01	0.24±0.00
Relative glycerol yield[a]	1.00	0.88±0.00	0.80±0.00	0.96±0.00
Residual sucrose (g·l^{-1})	<0.1	1.8±0.2	<0.1	1.5±0.1
Invertase activity (mmol·g $DW^{-1}·h^{-1}$)				
Total	128±7	284±35	265±22	N.D.
Extracellular	114±4	17±3	25±3	N.D.
Sucrose:H^+ transport capacity (mmol·g $DW^{-1}·h^{-1}$)	0.43±0.09	1.74±0.03	20.8±0.1	<0.3
Carbon recovery (%)[b]	96±0	97±1	100±1	97±1

N.D. not determined

[a]Yield relative to that of reference strain CEN.PK113-7D

[b]Calculations of the carbon recovery were based on a carbon content of biomass of 48% (w/w).

The biomass yield of the i*SUC2* strain was only 6±2% lower than that of the *SUC2* reference strain (Table 5.3). This difference is much smaller than the 25% difference that was anticipated in case of a completely intracellular hydrolysis of sucrose. Consistent with the small difference of the biomass yields of the two strains, only a minor increase of the ethanol yield was observed in the engineered strain (Table 5.3). Strikingly, residual concentrations of sucrose in the chemostat cultures of the engineered strain IMI056 (1.8 $g \cdot l^{-1}$) were much higher than those in cultures of the reference strain CEN.PK113-7D (<0.1 $g \cdot l^{-1}$, Table 5.3). This observation suggested that suboptimal kinetics of sucrose transport across the plasma membrane prevented efficient sucrose metabolism via intracellular hydrolysis. Indeed, although 4-fold higher than in the reference strain, the sucrose-proton symport capacity of the i*SUC2* strain (1.74 mmol·g biomass$^{-1} \cdot h^{-1}$) was two-fold lower than the sucrose consumption rate in the chemostat cultures (3.15 mmol·g biomass$^{-1} \cdot h^{-1}$). Based on these observations, we hypothesized that, due to the suboptimal kinetics of sucrose transport, alternative pathways, involving (an) unknown extracellular sucrose-hydrolyzing enzyme(s) and/or (a) low-affinity facilitated transporter(s) for sucrose, successfully competed with the intracellular hydrolysis pathway. We therefore attempted to improve the kinetics of sucrose-proton symport in *S. cerevisiae*.

Laboratory evolution in chemostat cultures improves sucrose-transport kinetics and ethanol yield

Prolonged nutrient-limited growth of microorganisms in chemostat cultures exerts a strong selective pressure for spontaneous mutants with an improved affinity for that limiting nutrient (μ_{max}/K_s) [12]. For example, prolonged sugar-limited chemostat cultivation of wild-type and engineered *S. cerevisiae* strains was shown to lead to improved transport kinetics for glucose [10, 21, 32], maltose [30] and xylose [35]. To investigate whether this approach is applicable to improve sucrose-uptake kinetics, the i*SUC2* strain IMI056 was grown in long-term anaerobic sucrose-limited chemostat cultures.

Over 90 generations of sucrose-limited growth, the residual sucrose concentration in the chemostat cultures decreased from 2 $g \cdot l^{-1}$ to around 0.1 $g \cdot l^{-1}$ (Figure 5.1). Over the same period, the sucrose-proton symport capacity increased by an order of magnitude (Figure 5.1). A single-cell isolate (referred to as the 'evolved i*SUC2* strain' IMM007) was obtained from one of the chemostat cultures by plating on non-selective glucose-containing agar plates and used to

inoculate fresh chemostat cultures. Within 8 volume changes, these cultures had reached a low residual sucrose concentrations (<0.1 $g \cdot l^{-1}$) and a very high sucrose-proton symport capacity (20.8±0.1 $mmol \cdot g\ DW^{-1} \cdot h^{-1}$), as was observed for the long-term chemostat cultures (Figure 5.1 and Table 5.3). This experiment indicated that the observed changes were the result of (a) mutation(s) in the genome of the i*SUC2* strain rather than of a physiological adaptation.

Analysis of transport kinetics using sucrose-grown shake-flask cultures revealed that the K_m of sucrose transport was unchanged (6±1 mM for both IMI056 and IMM007). These similar K_m values are consistent with an improvement of sucrose-uptake kinetics that was solely caused by an increased capacity (V_{max}) of the previously described high-affinity sucrose transport system [51].

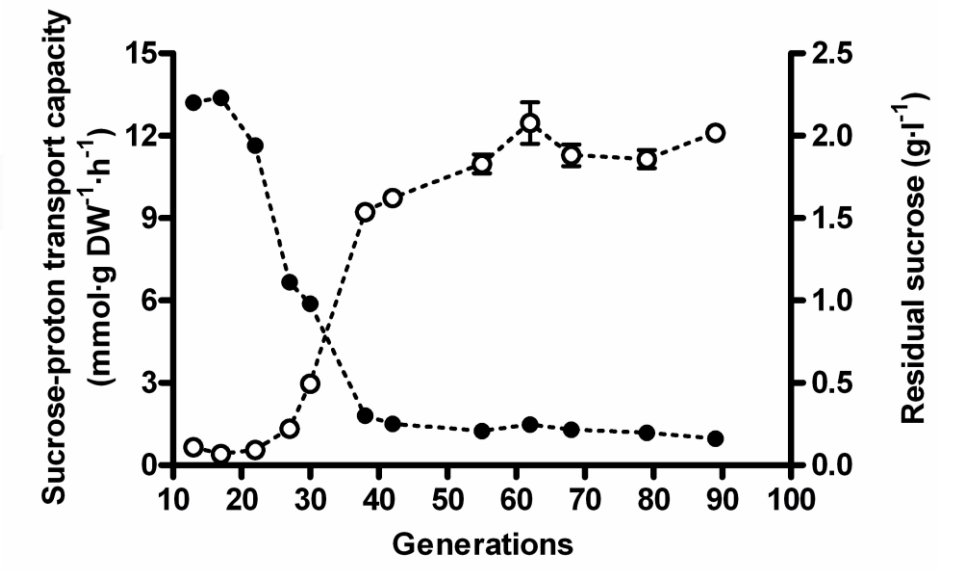

Figure 5.1. Long-term cultivation of *S. cerevisiae* IMI056 (i*SUC2*) in anaerobic sucrose-limited chemostat cultures at a dilution rate of 0.10 h^{-1}. Symbols: ●: residual sucrose concentration; ○: sucrose-proton symport capacity assayed in culture samples. An independent replicate experiment yielded similar results.

The biomass yield of the evolved i*SUC2* strain *S. cerevisiae* IMM007 on sucrose was 30±2% lower and its ethanol yield on sucrose was 11±2% higher than that of the *SUC2* reference strain CEN.PK113-7D (Table 5.3). These differences were in good agreement with the anticipated changes in case of a shift from extracellular hydrolysis to intracellular hydrolysis (25% decrease of biomass yield, 9% increase of ethanol yield; see above).

AGT1 plays a key role in the improved sucrose-transport kinetics of the evolved i*SUC2* strain

To investigate the molecular basis of the improved sucrose-uptake kinetics in the evolved i*SUC2* strain IMM007, its transcriptome was compared with that of the non-evolved i*SUC2* strain IMI056. Genome-wide transcriptome analysis was carried out with cell samples of both strains grown in anaerobic sucrose-limited chemostats at a dilution rate of 0.10 h^{-1}. The average coefficient of variation of the transcriptome data derived from at least duplicate, independent cultures did not exceed 20%. The level of *ACT1, ALG9, TAF10, TFC1* and *UBC6* transcripts, which are commonly used loading standards for Northern analysis [56], varied by less than 13% over the situations tested. A pair-wise comparison of the two strains yielded a total of 85 genes that were differentially transcribed based on the statistical criteria applied in this study (absolute fold difference ≥ 2, false discovery rate 0.5%; see Materials and methods). Out of the 85 differentially expressed genes 84 showed a higher expression level in the evolved i*SUC2* strain IMM007 relative to its parental non-evolved strain IMI056 and only one gene (YGR234W) exhibited a lower expression level.

Genes involved in carbohydrate metabolism and transport and, albeit less pronounced, in stress and stimulus responses were overrepresented among the differentially expressed genes (Table 5.4). In particular, 9 out of 11 genes involved in maltose metabolism showed a strongly increased transcript level in the evolved strain (Table 5.4). Comparison of the relative transcript levels of the differentially expressed 'carbohydrate metabolism and transport' genes between the non-evolved and evolved i*SUC2* strain, showed higher transcript levels of genes involved in carbohydrate metabolism and, in particular, of maltose metabolism in the evolved strain. This general deregulation resembled the relative transcript levels in aerobic chemostat cultures of the reference strain CEN.PK113-7D grown under glucose and maltose limitation, respectively (Figure 5.2).

Among the genes with an increased transcript level in the evolved i*SUC2* strain, *AGT1* encodes an α-glucoside-proton symporter whose substrates include both maltose and sucrose [52]. To investigate the involvement of *AGT1* in the improved sucrose-transport kinetics, it was deleted in the evolved *iSUC2* strain IMM007. A transformant that showed correct integration of the *AGT1* gene replacement cassette still exhibited about half of the sucrose-proton symport capacity of the evolved strain (data not shown). Diagnostic PCR on genomic DNA showed that a duplication of the *AGT1* gene had occurred in the evolved i*SUC2* strain (data not shown). When both copies of *AGT1* in the evolved i*SUC2* strain were deleted, a drastic decrease of its sucrose-proton symport activity was observed (Table 5.3).

When both *AGT1* copies in the evolved i*SUC2* strain IMM007 were deleted, the resulting strain IMM009 exhibited the same yields of biomass, ethanol and glycerol as the *SUC2* reference strain CEN.PK113-7D (Table 5.3). This correspondence is consistent with an essential role of the sucrose transporter Agt1p in enabling intracellular sucrose hydrolysis and thus efficient sucrose fermentation [3, 5]. Residual sucrose concentrations in chemostat cultures of the *agt1*Δ/*agt1*Δ strain IMM009 were much higher than those in the *SUC2* reference strain CEN.PK113-7D and comparable to those in the unevolved i*SUC2* strain IMI056 (Table 5.3). The difference with the *SUC2* reference strain can be attributed to the high extracellular invertase activity in the latter.

Sequencing of *AGT1* in the original i*SUC2* strain IMI056, and in the evolved i*SUC2* strains IMM007 and IMM008 (*agt1*Δ) revealed no mutations, neither in its coding region nor in an 800 bp upstream region. This result indicates that the increased expression of *AGT1* was due to a second-site mutation, for example in a transcriptional regulator gene (e.g. *MALx3*). Such a mutation could then also be responsible for the upregulation of other genes involved in disaccharide metabolism in the evolved i*SUC2* strain (Table 5.4, Figure 5.2). In view of anticipated problems in the assembly of the multiple *MAL* loci from short-read sequencing data and in the absence of a completely assembled and annotated reference sequence of *S. cerevisiae* CEN.PK113-7D, we have refrained from resequencing the genome of the evolved i*SUC2* strain.

Table 5.4 Gene Ontology functional categories overrepresented in the set of genes differentially expressed (FC $\geq |2|$, FDR = 0.5%) in the evolved i*SUC2* strain *S. cerevisiae* IMM007 relative to the unevolved i*SUC2* strain IMI056.

GO Complete category	k in n[a]	*p*-value[b]	Genes
Maltose metabolic process	9 out of 11	$1.7 \cdot 10^{-11}$	*MPH2, MPH3, FSP2, IMA5, AGT1, MAL31, MAL12, MAL32, MAL13*
Carbohydrate metabolic process	20 out of 244	$1.6 \cdot 10^{-9}$	*MPH2 , MPH3, FSP2, IMA5, AGT1, MAL31, MAL12, MAL32,* YHR210C*, GRE3, SOL4, AMS1, MIG2, GIP2, GLC3, TPS2, TPS1, GPH1, TSL1, MAL13*
Disaccharide metabolic process	12 out of 24	$1.5 \cdot 10^{-13}$	*MPH2, MPH3, FSP2, IMA5, AGT1, TPS2, MAL31, MAL12, MAL32, TPS1, TSL1, MAL13*
Trehalose metabolic process	3 out of 10	$2.5 \cdot 10^{-4}$	*TPS2, TPS1, TSL1*
Trehalose biosynthetic process	3 out of 7	$7.4 \cdot 10^{-5}$	*TPS2, TPS1, TSL1*
Carbohydrate transport	5 out of 41	$1.8 \cdot 10^{-4}$	*MPH2, MPH3, AGT1, MAL31, HXT2*
Cellular carbohydrate metabolic process	18 out of 199	$4.3 \cdot 10^{-9}$	*MPH2, MPH3, FSP2, IMA5, GRE3, AGT1, SOL4, AMS1, GIP2, GLC3, TPS2, MAL31, MAL12, MAL32, TPS1, GPH1, TSL1, MAL13*
Disaccharide biosynthetic process	3 out of 7	$7.4 \cdot 10^{-5}$	*TPS2, TPS1, TSL1*
Response to stimulus	26 out of 911	$4.7 \cdot 10^{-4}$	*AHP1, MNN4, UGA2, GRE3, DOG1, DOG2, ASK10, STF2, MIG2, HSP12, TSA2, TPS2, HSP30, FRM2, SSE2, TPS1, HSP26, SSA3, DDR2, GRE2, HUG1, DDR48, ALD2, ALD3, RAD52, TSL1*
Response to stress	24 out of 621	$1.1 \cdot 10^{-5}$	*AHP1, MNN4, UGA2, GRE3, DOG1, DOG2, ASK10, STF2, HSP12. TSA2, TPS2, HSP30, SSE2, TPS1, HSP26, SSA3, DDR2, GRE2, HUG1, DDR48, ALD2, ALD3, RAD52, TSL1*

[a] k represents the number of gene from a GO category in the differentially expressed genes and n represents the number of genes from the same GO category in the whole genome.

[b] Enrichment analysis *p*-value according to Fischer exact statistics.

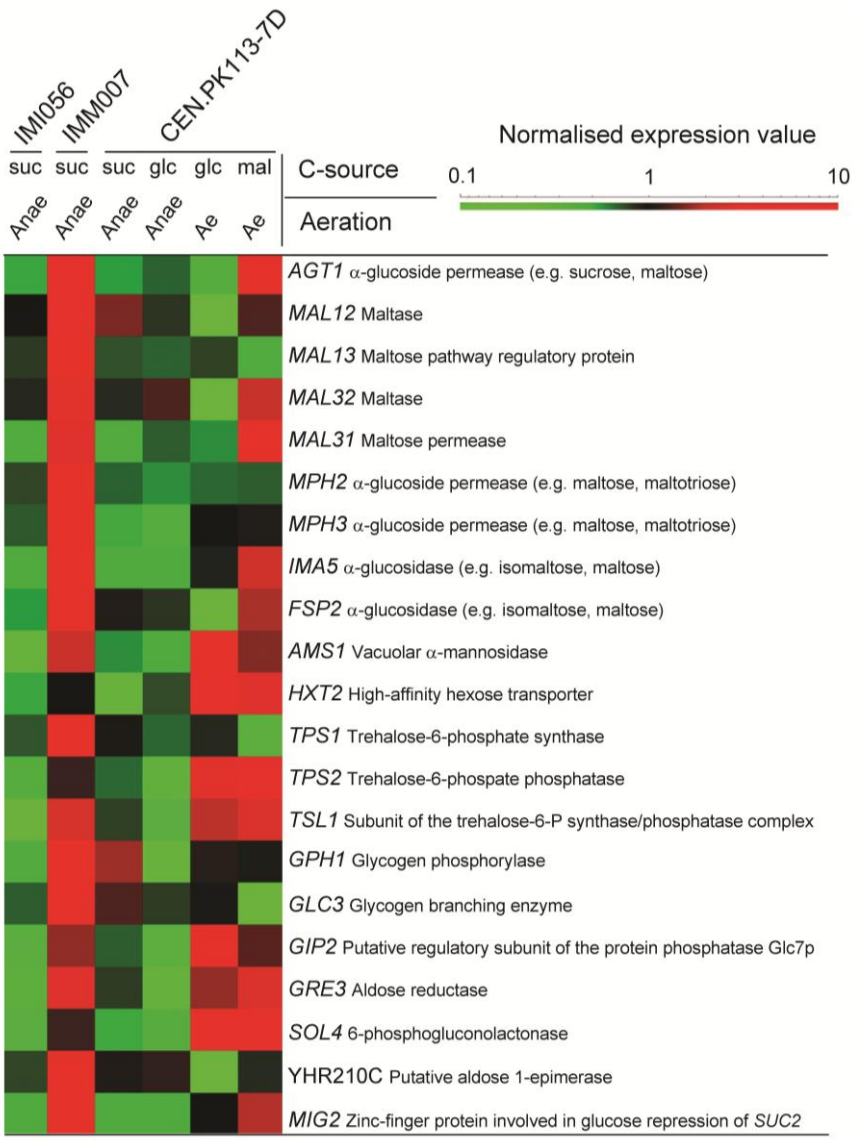

Figure 5.2 Heat map of genes in GO categories related to carbohydrate metabolism with significantly higher transcript level in the evolved i*SUC2* strain *S. cerevisiae* IMM007 as compared to its parental strain IMI056 (Table 5.4). Transcript levels are compared to those of the reference strain CEN.PK113-7D (*SUC2*) grown in anaerobic sucrose-limited cultures (this study), in anaerobic glucose-limited chemostat cultures [54] and in aerobic glucose- and maltose-limited chemostat cultures [15].

Discussion

Topology, kinetics and energetics of sucrose metabolism in yeast

Relocation of the *SUC2*-encoded invertase to the cytosol was, in itself, not sufficient to achieve the changes in growth stoichiometry that were predicted for intracellular sucrose hydrolysis by *S. cerevisiae*. This suggested that a low extracellular sucrose-hydrolyzing activity of unknown identity competed with an insufficient activity of the *AGT1*-encoded sucrose-proton symporter. Subsequent laboratory evolution in sucrose-limited chemostats did not lead to an increased activity of extracellular sucrose hydrolysis but, instead, led to improved kinetics of sucrose-proton symport (Table 5.3, Figure 5.1). Apparently, under sucrose limitation, the selective advantage of a higher substrate affinity, in this case conveyed by increased transport capacity, outweighed the lower ATP yield resulting from the combination of active transport and intracellular hydrolysis of sucrose. It is interesting to investigate whether the same evolutionary engineering strategy can be applied to improve uptake kinetics of engineered *S. cerevisiae* strains for other industrially relevant disaccharides, such as lactose and cellobiose [19, 22, 45]. In the latter case, a high affinity of cells for the disaccharide may also help to alleviate cellobiose inhibition of cellulases [37] during simultaneous saccharification and fermentation processes.

In principle, improved kinetics of sucrose transport can also be achieved by targeted overexpression of *AGT1* or heterologous genes encoding sucrose-proton symporters, for example from yeasts that naturally hydrolyze sucrose intracellularly [33]. Irrespective of the method by which the transport kinetics of disaccharide metabolism are engineered, care should be taken to avoid an overcapacity as this may cause substrate-accelerated death when the yeast cells are exposed to high sugar concentrations, as demonstrated for *S. cerevisiae* strains that overexpress the maltose-proton symporters [30].

Maltase (α-glucosidase, EC 3.1.2.20) has been shown to be responsible for intracellular sucrose hydrolysis in some naturally occurring sucrose-metabolizing yeasts [33]. In a separate study, we have shown that expression of maltase (Malx2p), combined with the deletion of *SUC2*, provides an alternative strategy for changing the topology of sucrose metabolism in *S. cerevisiae* (M. Dario *et al.*, manuscript in preparation).

From academic proof of principle to industrial application

This study provides an academic proof of principle for improvement of ethanol yields in anaerobic sucrose-grown cultures of *S. cerevisiae* by engineering the topology of sucrose metabolism. The observed increase in ethanol yield in the evolved i*SUC2* strain was consistent with the theoretical prediction (9%, Equation 5.2). The yield improvement that can be achieved in industry with the i*SUC2* strategy depends on the specific growth rate and biomass yield. Since the specific growth rate under industrial conditions is lower than the value of 0.10 h^{-1} used in this study, a larger fraction of the sucrose will already be dissimilated via alcoholic fermentation due to the increased impact of maintenance-energy requirement at low specific growth rates [7, 43]. This would leave less room for improvement of the ethanol yield by changing the topology of sucrose metabolism (i.e. a higher $Y_{SucE,extra}$ in Equation 5.2). However, at a specific growth rate as low as 0.025 h^{-1}, still a significant increase of the ethanol yield of 3% is predicted (based on an $Y_{SucE,extra}$ of 3.56 mol·mol sucrose^{-1}, derived from an ethanol yield of 1.78 mol·mol glucose^{-1} [7]).

In addition to improved ethanol yields, intracellular sucrose hydrolysis may have other advantages. For example, production of extracellular invertase can allow growth on glucose and fructose of other microorganisms that lack invertase, including invertase-negative yeasts [26, 27]. Moreover, extracellular accumulation of fructose can cause problems in industrial processes due to slower fructose utilization by industrial *S. cerevisiae* strains, resulting in residual fructose at the end of the cultivation and reduced ethanol yields [6]. Indeed, anaerobic batch cultures on sucrose of the evolved i*SUC2* strain showed a drastic reduction of extracellular glucose and fructose accumulation as compared to the *SUC2* reference strain (data not shown).

In view of the high impact of feedstock costs on the process economy of bioethanol production [38], industrial implementation of the i*SUC2* strategy offers an attractive perspective. Hitherto, implementation of genetically modified yeast strains in cane-sugar based ethanol production still presents challenges, for example due to the need to maintain asepsis. However, as more strain engineering approaches become available to improve key yeast characteristics, such as reduction of byproduct formation [9, 28, 29, 40] and improvement of ethanol tolerance [64], the incentive for introduction of engineered strains will grow. Further research in our groups will therefore focus on implementation of intracellular sucrose hydrolysis in industrial ethanol

production strains and evaluation of their performance under industrial process conditions.

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Summary

of the PhD thesis
'Metabolic engineering of free-energy (ATP) conserving reactions in *Saccharomyces cerevisiae*'

Metabolic engineering – the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell – has enabled the industrial production of a wide variety of biological molecules from renewable resources. Microbial production of fuels and chemicals thereby provides an alternative to oil-based production. To compete with petrochemical production, not only the kinetics of product formation, but also the product yield needs to be optimized. The maximum product yield is determined by the stoichiometry of the product pathway with respect to (i) the conversion of substrate into product on a molar or carbon basis, (ii) the production or consumption of redox cofactors (e.g. NAD(P)(H)) and (iii) the efficiency of free-energy conservation in the form of ATP. Whereas reduction of byproduct formation, modification of redox-cofactor balances and optimization of the stoichiometry of product formation is studied in many laboratories, the ATP yield of a product pathway is often overlooked, although it is of paramount importance for the product yield that can be obtained. Product pathways that do not result in net conservation of free energy or even demand a net input of free energy in the form of ATP, require an additional pathway to supply the ATP needed for product formation, cellular maintenance and/or growth. In industrial processes, this role is usually fulfilled by respiration. However, aeration of industrial-scale fermentations is expensive and results in dissimilation of part of the substrate to CO_2 at the expense of product formation. On the other hand, product pathways with a high ATP yield may result in excess biomass formation, which also goes at the expense of the product yield. The importance of the ATP yield on product formation and opportunities for improvement through metabolic engineering of free-energy (ATP) conservation have been illustrated in **chapter 1**.

An illustrative example of the importance of increased free-energy (ATP) conservation is lactic acid production by engineered *Saccharomyces cerevisiae* strains. Conversion of glucose to lactic acid is equivalent to ethanol production in terms of NAD(H) and ATP yield. Whereas ethanol can be produced efficiently under

anaerobic conditions, engineered 'homolactic' *S. cerevisiae* strains cannot grow under anaerobic conditions, presumably because export of one molecule of lactic acid requires the net hydrolysis of one molecule of ATP. This results in a 'zero-ATP pathway', which precludes efficient lactic acid production under anaerobic conditions. Therefore, the goal of this PhD project was to study concepts for improvement of free-energy conservation (ATP-yield) that have the potential to enable anaerobic lactic acid production by *S. cerevisiae*.

Hitherto, the exact mechanism of and the gene(s) responsible for lactic acid export in *S. cerevisiae* are unknown. Jen1p is the only *S. cerevisiae* lactate transporter reported in literature and is required for import of and growth on lactic acid. However, deletion of *JEN1* did not affect lactate export (my unpublished results). To identify alternative lactate transporters, a *jen1*Δ strain was evolved for growth on lactic acid (**chapter 2**). In two independent evolution experiments, Jen1p-independent growth on lactate was observed within 100 generations of evolution, with maximum specific growth rates of 0.14 and 0.18 h^{-1} for single-cell lines IMW004 and IMW005, respectively. To identify the genetic changes that are responsible for the improved phenotypes, transcriptome analysis and whole-genome resequencing were combined. Whereas transcriptome analysis did not provide valuable leads, whole-genome resequencing showed different single-nucleotide changes (C755G/Leu219Val in IMW004 and C655G/Ala252Gly in IMW005) in the acetate transporter gene *ADY2*. Detailed analysis of mRNA expression levels and depth of coverage of DNA sequencing data indicated a triplication of a part of chromosome III. Subsequent karyotyping, gene deletion studies and diagnostic PCR showed that in IMW004 an additional isochromosome III (ca. 475 kb), which contained two extra copies of *ADY2*^{C755G}, was formed via crossover between the Ty1-type retrotransposons *YCLWΔ15* and *YCRCΔ6*. Knockout of all copies of the mutated *ADY2* alleles in IMW004 and IMW005 abolished the ability to grow on lactic acid and reverse metabolic engineering of the mutated *ADY2* alleles in a *jen1*Δ *ady2*Δ strain resulted in efficient growth on lactate (μ_{max} 0.14 h^{-1} for Ady2p^{Leu219Val} and 0.12 h^{-1} for Ady2p^{Ala252Gly}), thereby confirming the crucial role of the mutated *ADY2* alleles for lactic acid transport. Expression of the native *ADY2* allele in a *jen1*Δ *ady2*Δ strain already resulted in slow growth (μ_{max} ca. 0.02 h^{-1}) on lactic acid, showing that the native Ady2p can also transport lactate, albeit at a low rate. Furthermore, the wild-type and mutated Ady2p alleles were able to transport

pyruvate. Subsequently, the role of Ady2p and two close homologues (Ato2p and Ato3p) in lactate and pyruvate acid export was investigated (my unpublished results). Deletion of *ADY2* in a *jen1*Δ strain did not lower the lactate or pyruvate export rate. Additional deletion of the lactate transporter candidate genes *ATO2* and *ATO3* in a *jen1*Δ *ady2*Δ strain did not lower the lactate export rate, either. These data indicate the presence of at least one unidentified plasma-membrane lactate transporter in *S. cerevisiae*.

The ATP requirement for export prevents efficient anaerobic lactic acid production by *S. cerevisiae*. Moreover, a net input of free energy is probably required for product export when lactate is produced at industrially desirable high extracellular product concentrations and low pH. Alternative strategies to obtain extra ATP from the conversion of substrate into product are therefore highly relevant for anaerobic production of lactic acid and other 'zero-ATP products' by *S. cerevisiae*. One such approach was investigated in **chapter 3**, where hydrolysis of maltose was replaced by maltose phosphorolysis to increase the ATP yield during alcoholic fermentation on this disaccharide. In wild-type *S. cerevisiae*, maltose enters the cell via a proton-symport mechanism and is subsequently hydrolyzed into two molecules of glucose. To maintain intracellular pH homeostasis, the imported proton has to be exported by the plasma-membrane H^+-ATPase, which, in *S. cerevisiae*, has a stoichiometry of 1 H^+ per hydrolyzed ATP. Anaerobic conversion of maltose via the Embden-Meyerhof-Parnas glycolytic pathway to ethanol therefore only yields 3 ATP per maltose. In many prokaryotes, maltose is cleaved with inorganic phosphate into glucose and glucose-1-phosphate, a reaction catalyzed by maltose phosphorylase. The formed glucose-1-phosphate can be converted by a phosphoglucomutase to the glycolytic intermediate glucose-6-phosphate. As a result one less ATP is hydrolyzed per maltose in the hexokinase reaction to phosphorylate glucose to glucose-6-phosphate. Replacing the hydrolysis of maltose with phosphorolytic cleavage therefore theoretically increases the anaerobic ATP yield from 3 to 4 ATP per maltose. To test this concept, the native maltose metabolism genes in *S. cerevisiae* were first deleted. The resulting knockout strain showed no maltose-transport activity and a very low residual maltase activity (0.03 μmol·mg protein^{-1} ·min^{-1}). Expression of a maltose phosphorylase gene from *Lactobacillus sanfranciscensis* and the *S. cerevisiae MAL11* maltose transporter gene in this knockout strain resulted in relatively slow growth ($\mu_{aerobic}$ 0.09±0.03 h^{-1}),

presumably due to an absence of β-phosphoglucomutase activity (<0.005 μmol·min^{-1}·mg protein^{-1}). Co-expression of a β-phosphoglucomutase from *Lactococcus lactis* was required for efficient maltose utilization via the maltose phosphorylase route ($\mu_{aerobic}$ 0.21±0.01 h^{-1}, $\mu_{anaerobic}$ 0.10±0.00 h^{-1}). Quantitative analysis of the resulting strain showed that replacing maltose hydrolysis with phosphorolysis increased the anaerobic biomass yield on maltose by 26%. However, due to the energy cost of maltose import, anaerobic maltose catabolism via the maltose phosphorylase route still only yields 2 ATP per hexose equivalent and is therefore energetically equal to glucose catabolism. In theory, this ATP yield can be further increased to above 2 ATP per hexose equivalent by introduction of a maltotriose phosphorylase and using maltotriose as substrate or introduction of a maltose transporter with a uniporter mechanism. Unfortunately, despite multiple attempts, neither the functional expression of a heterologous maltotriose phosphorylase nor the functional expression of a maltose uniporter was achieved in this project. Ultimately, anaerobic sugar metabolism that yields more than 2 ATP per hexose equivalent may enable anaerobic production of compounds whose redox-closed formation currently has a very low or zero ATP yield.

Another mechanism to improve free-energy (ATP) conservation is the introduction of a plasma-membrane H$^+$-ATPase with an increased H$^+$/ATP stoichiometry. In literature, two isoforms (Ser800Ala and Glu803Gln) of the plasma-membrane H$^+$-ATPase Pma1p were described that showed an increased *in vitro* H$^+$/ATP stoichiometry. In **chapter 4**, the *in vivo* H$^+$/ATP stoichiometry of these isoforms was investigated by measuring the anaerobic biomass yield on maltose. In theory, an increased H$^+$/ATP stoichiometry will increase the biomass yield on maltose due to a decreased energy-requirement for export of the proton imported with maltose. To characterize the *in vivo* H$^+$/ATP stoichiometry of the Ser800Ala and Glu803Gln isoforms of Pma1p, the wild-type *PMA1* allele was replaced by the corresponding *PMA1* mutant alleles in a *pma2*Δ background and the anaerobic biomass yields on maltose were compared to those of an isogenic *PMA1 pma2*Δ reference strain. No differences in biomass yield were observed, showing that introduction of these mutations in *PMA1* did not increase the *in vivo* H$^+$/ATP stoichiometry and that increased *in vitro* proton stoichiometries do not guarantee *in vivo* 'hypercoupling'. The method presented in this study

provides a useful tool in the continuing search for plasma-membrane H^+-ATPase isoforms with an *in vivo* H^+/ATP stoichiometry above one.

'Zero-ATP processes' such as lactic and malic acid production by engineered *S. cerevisiae* strains could benefit from the strategies for increased free-energy (ATP) conservation that were investigated in chapters 3 and 4. Conversely, industrial processes such as bio-ethanol production, in which product formation is coupled to a net production of ATP, might benefit from decreased free-energy conservation, since ATP availability enables biomass formation and thereby decreases the product yield. In that case, decreasing free-energy (ATP) conservation presents an interesting strategy to increase the product yield. In **chapter 5**, this concept was investigated for alcoholic fermentation of sucrose, which is the feedstock for roughly half of the global bioethanol production. In *S. cerevisiae*, the disaccharide sucrose is primarily hydrolyzed outside the cell, followed by uptake of the monomeric sugars glucose and fructose via facilitated diffusion. Subsequent conversion to ethanol via the Embden-Meyerhof-Parnas pathway yields 4 ATP per sucrose. Alternatively, sucrose can be imported via a sucrose-proton-symport mechanism and hydrolyzed inside the cell. Extrusion of the imported proton via the plasma-membrane H^+-ATPase costs 1 ATP and intracellular sucrose hydrolysis therefore only yields 3 ATP per sucrose. As a result of the ATP cost of sucrose import, relocating sucrose hydrolysis from outside to inside the cell should, in theory, decrease the biomass yield by 25% and increase the ethanol yield by 9%. This hypothesis was tested by engineering the promoter and 5' coding sequences of the invertase-encoding gene *SUC2*, which resulted in predominant (94%) cytosolic localization of sucrose hydrolysis. However, in anaerobic sucrose-limited chemostats, this engineered i*SUC2*-strain showed an only 4% increased ethanol yield. Furthermore, high residual sucrose concentrations (ca. 2 $g \cdot l^{-1}$) indicated suboptimal sucrose-transport kinetics. To improve sucrose-uptake affinity, the i*SUC2*-strain was subjected to 90 generations of laboratory evolution in an anaerobic sucrose-limited chemostat. This evolution resulted in a 20-fold decrease of residual sucrose concentrations and a 10-fold increase of the sucrose-transport capacity. A single-cell isolate showed an 11% increased ethanol yield and 30% decreased biomass yield on sucrose in chemostat cultures when compared to an isogenic *SUC2* reference strain. Transcriptome analysis of this evolved strain revealed elevated expression of *AGT1*, encoding a disaccharide-proton symporter, and other maltose-related

genes. Knockout studies and diagnostic PCR showed that *AGT1* was duplicated during evolution. After deletion of both copies of *AGT1*, the residual sucrose concentration and transport capacity reverted to that of the unevolved i*SUC2*-strain and the biomass and ethanol yield reverted to that of the *SUC2*-strain. The concept of relocating sucrose hydrolysis to increase the ethanol yield has been transferred to industrial strain backgrounds and the performance of the resulting strains under industrial conditions is being investigated.

The research described in this thesis illustrates that the efficiency of free-energy (ATP) conservation is important for efficient production of low-molecular weight compounds, such as lactic acid and ethanol. For product pathways with excess ATP and biomass formation, a widely applicable strategy (relocating sucrose hydrolysis) was presented to decrease free-energy (ATP) conservation and thereby increase the product yield. On the other hand, the efficiency of free-energy (ATP) conservation was increased by expression of a maltose phosphorylase, which forms the basis for further development of these strategies towards increasing the ATP yield of many industrial target pathways and specifically to potentially enable efficient anaerobic homolactate production with *S. cerevisiae*.

Samenvatting

van het proefschrift
'Metabolic engineering van vrije-energie (ATP) conserverende reacties in *Saccharomyces cerevisiae*'

Metabolic engineering – het verbeteren van cellulaire processen door manipulatie van enzymatische, transport- en regulerende processen in levende cellen – heeft de industriële productie van een breed scala aan biologische moleculen vanuit hernieuwbare grondstoffen mogelijk gemaakt. De microbiële productie van brandstoffen en chemicaliën biedt daarmee een alternatief voor de traditionele, op olie gebaseerde productieprocessen. Om te kunnen concurreren met petrochemische productie moet niet alleen de snelheid van microbiële productvorming, maar ook de productopbrengst worden geoptimaliseerd. De maximale opbrengst van product uit substraat wordt bepaald door de stoichiometrie van de productvormingsroute, met betrekking tot (i) de omzetting van substraat naar product op een molaire of koolstofbasis, (ii) de productie of consumptie van redox-cofactoren (bijvoorbeeld NAD(P)(H)) en (iii) de efficiëntie van vrije-energieconservering in de vorm van ATP. Terwijl vermindering van bijproductvorming, modificatie van de redox-cofactorbalans en optimalisatie van de stoichiometrie van productvorming in veel laboratoria worden bestudeerd, wordt de energiehuishouding vaak over het hoofd gezien. De energiehuishouding is echter van groot belang voor de productopbrengst die kan worden behaald. Productvormingsroutes die niet leiden tot conservering van vrije energie (ATP) of waarvoor zelfs een investering van vrije energie in de vorm van ATP nodig is, vereisen namelijk een andere stofwisselingsroute om de ATP te leveren voor productvorming, onderhoud en/of groei van de cellen. In industriële processen wordt deze rol vaak vervuld door ademhaling. Beluchting van fermentaties op industriële schaal is echter duur en leidt tevens tot verbranding van een deel van het substraat tot koolstofdioxide, wat ten koste gaat van productvorming. Andersom leiden productvormingsroutes met een hoge ATP opbrengst tot overmatige biomassavorming, wat ook weer ten koste gaat van productvorming. Het belang van vrije-energie (ATP) conservering op productvorming en mogelijkheden om de energiehuishouding aan te passen, zijn besproken in **hoofdstuk 1**.

Een sprekend voorbeeld van het belang van conservering van vrije energie (ATP) is de productie van melkzuur door genetisch gemodificeerde *Saccharomyces cerevisiae* stammen. De omzetting van glucose naar melkzuur is qua NAD(H)- en ATP-opbrengst gelijk aan ethanolvorming. Terwijl ethanol efficiënt kan worden geproduceerd onder anaërobe condities, kunnen gemodificeerde 'homolactaat'-stammen van *S. cerevisiae* echter niet groeien onder anaërobe omstandigheden, vermoedelijk omdat export van één molecuul melkzuur uit de cel de netto hydrolyse van één molecuul ATP vereist. Dit resulteert in een ATP-neutrale stofwisselingsroute, hetgeen efficiënte productie van melkzuur onder anaërobe condities verhindert omdat geen ATP overblijft voor groei en onderhoud van de cellen. Het doel van dit promotieonderzoek was daarom het bestuderen van concepten die de vrije-energieconservering (ATP-opbrengst) verbeteren en daarmee, in potentie, anaërobe melkzuurproductie door *S. cerevisiae* mogelijk maken.

Tot op heden zijn het exacte mechanisme van melkzuurexport en het gen/de genen verantwoordelijk voor melkzuurexport in *S. cerevisiae* onbekend. Jen1p is de enige in de literatuur bekende *S. cerevisiae* melkzuurtransporter. Deze transporter is noodzakelijk voor import van melkzuur en daarmee voor groei op melkzuur. Deletie van *JEN1* heeft echter geen invloed op melkzuurexport (mijn ongepubliceerde resultaten). Om alternatieve melkzuurtransporters te ontdekken, is in **hoofdstuk 2** een *jen1*Δ stam geëvolueerd voor groei op melkzuur. In twee onafhankelijke evolutie-experimenten resulteerde dit binnen 100 generaties in Jen1p-onafhankelijke groei op melkzuur, met een maximale specifieke groeisnelheid van 0,14 h^{-1} en 0,18 h^{-1} voor de geïsoleerde kolonies IMW004 en IMW005. Om de genetische veranderingen die verantwoordelijk zijn voor het veranderde fenotype te identificeren, is een transcriptoomanalyse uitgevoerd en bovendien het hele genoom van de geëvolueerde stammen 'gesequenced'. Transcriptoomanalyse gaf geen waardevolle aanwijzingen, maar genoomsequencing leidde tot de ontdekking van mutaties in het azijnzuurtransporter-gen *ADY2* (C755G/Leu219Val in IMW004 en C655G/Ala252Gly in IMW005). Gedetailleerde analyse van mRNA-expressieniveaus en DNA-sequentiegegevens leverde aanwijzingen voor een triplicatie van een deel van chromosoom III. Via chromosoomanalyse, gendeletiestudies en diagnostische polymerasekettingreacties werd aangetoond dat in IMW004 een isochromosoom III (circa 475 kb) met twee extra kopieën

van *ADY2*^{C755G} was gevormd via recombinatie tussen de Ty1-type retrotransposons *YCLWΔ15* en *YCRCΔ6*. Na deletie van alle kopieën van de gemuteerde *ADY2*-allelen in IMW004 en IMW005 konden deze stammen niet meer op melkzuur groeien. Expressie van de gemuteerde *ADY2*-allelen in een *jen1*Δ *ady2*Δ stamachtergrond resulteerde in efficiënte groei op melkzuur (μ_{max} 0,14 h^{-1} voor Ady2p^{Leu219Val} en 0,12 h^{-1} voor Ady2p^{Ala252Gly}), hetgeen de cruciale rol van de gemuteerde *ADY2*-allelen voor melkzuurtransport bevestigde. Expressie van het normale *ADY2*-allel in een *jen1*Δ *ady2*Δ stamachtergrond resulteerde ook al tot (langzame) groei op melkzuur (μ_{max} circa 0,02 h^{-1}), waaruit bleek dat het normale Ady2-eiwit ook lactaat kan transporteren, zij het op een lage snelheid. Daarnaast bleek dat zowel wild-type Ady2p als de gemuteerde Ady2p-isovormen in staat zijn om pyrodruivenzuur te transporteren. Vervolgens is de rol van Ady2p en twee nauw verwante transporters (Ato2p en Ato3p) in melkzuurexport onderzocht (mijn ongepubliceerde resultaten). Deletie van *ADY2* in een *jen1*Δ stamachtergrond verlaagde de snelheid van melkzuur- en pyrodruivenzuurexport niet en ook de extra deletie van *ATO2* en *ATO3* in een *jen1*Δ *ady2*Δ stamachtergrond leidde niet tot een verlaging van de melkzuurexportsnelheid. Deze waarnemingen duiden erop dat er ten minste één ongeïdentificeerde melkzuurtransporter actief is in het plasmamembraan van *S. cerevisiae*.

De ATP-behoefte voor export verhindert efficiënte anaërobe productie van melkzuur door *S. cerevisiae*. Deze vrije-energie-investering is echter waarschijnlijk noodzakelijk voor productexport wanneer melkzuur wordt geproduceerd onder industriële condities, dat wil zeggen bij hoge extracellulaire productconcentraties en lage pH. Alternatieve strategieën om extra ATP te verkrijgen vanuit de omzetting van substraat naar product zijn daarom zeer relevant voor anaërobe productie van melkzuur en andere 'ATP-neutrale' producten door *S. cerevisiae*. Een dergelijk concept is onderzocht in **hoofdstuk 3**, waar de hydrolyse van maltose is vervangen door fosforolyse om de ATP-opbrengst tijdens alcoholische vergisting van deze disaccharide te verhogen. In normale *S. cerevisiae* stammen wordt maltose de cel in getransporteerd via een proton-symport mechanisme en vervolgens gehydrolyseerd tot twee moleculen glucose. Voor intracellulaire pH homeostase dient het geïmporteerde proton geëxporteerd te worden door het plasmamembraan H^+-ATPase. Dit membraaneiwit heeft in *S. cerevisiae* een stoichiometrie heeft van één proton per ATP. Anaërobe omzetting

van maltose naar ethanol via de Embden-Meyerhof-Parnas glycolytische stofwisselingsroute levert daarom netto maar 3 ATP op per maltose. In veel prokaryoten wordt maltose door het enzym maltosefosforylase gesplitst, waarbij maltose met anorganisch fosfaat wordt omgezet tot glucose en glucose-1-fosfaat. Het gevormde glucose-1-fosfaat kan vervolgens worden omgezet door een fosfoglucomutase naar het glycolytische intermediair glucose-6-fosfaat. Dit heeft tot gevolg dat er 1 ATP minder hoeft te worden geïnvesteerd in de omzetting van maltose naar 2 moleculen glucose-6-fosfaat. Het vervangen van de hydrolyse van maltose door een fosforolytische splitsing verhoogt daarom theoretisch de netto anaërobe ATP-opbrengst van 3 naar 4 ATP per maltose. Om dit concept te testen, zijn eerst de genen van het maltosemetabolisme in *S. cerevisiae* verwijderd. De resulterende deletiestam vertoonde geen maltosetransportactiviteit meer en slechts een zeer lage residuele maltase-activiteit (0,03 $\mu mol \cdot min^{-1} \cdot mg\ eiwit^{-1}$). Expressie van het maltosefosforylase-gen uit *Lactobacillus sanfranciscensis* en het *S. cerevisiae MAL11* maltosetransporter-gen in deze deletiestam resulteerde slechts in relatief langzame groei ($\mu_{aëroob}$ 0,09±0,03 h^{-1}), waarschijnlijk door een gebrek aan β-fosfoglucomutase-activiteit (<0,005 $\mu mol \cdot min^{-1} \cdot mg\ eiwit^{-1}$). Co-expressie van een β-fosfoglucomutase uit *Lactococcus lactis* was noodzakelijk voor efficiënte omzetting van maltose via de maltosefosforylaseroute ($\mu_{aëroob}$ 0,21±0,01 h^{-1}, $\mu_{anaëroob}$ 0,10±0,00 h^{-1}). Kwantitatieve analyse van de groei van genetisch gemodificeerde giststammen toonde aan dat het vervangen van maltosehydrolyse door fosforolyse de biomassaopbrengst met 26% verhoogde. Echter, vanwege de (indirecte) ATP-behoefte voor import van maltose levert anaëroob maltosekatabolisme via de maltosefosforylaseroute slechts 2 ATP per hexose-equivalent op, hetgeen equivalent is aan de ATP-opbrengst uit glucosekatabolisme. Theoretisch kan deze ATP-opbrengst verder worden verhoogd tot boven 2 ATP per hexose-equivalent door een maltotriosefosforylase tot expressie te brengen en maltotriose te gebruiken als substraat óf door expressie van een maltosetransporter met een uniportmechanisme. Helaas is, ondanks meerdere pogingen, functionele expressie van een heterologe maltotriosefosforylase en/of een maltose-uniporter in *S. cerevisiae* niet bereikt in dit project. Anaëroob suikermetabolisme met een netto opbrengst van meer dan 2 ATP per hexose equivalent kan uiteindelijk anaërobe productie mogelijk maken van producten waarvan de redox-neutrale omzetting op dit moment zeer weinig of geen ATP oplevert.

Een andere manier om vrije-energieconservering te verbeteren is de introductie van een plasmamembraan H^+-ATPase met een verhoogde H^+/ATP stoichiometrie. In de literatuur zijn twee isovormen (Ser800Ala en Glu803Gln) van het plasmamembraan H^+-ATPase Pma1p beschreven met een verhoogde *in vitro* H^+/ATP stoichiometrie. In **hoofdstuk 4** is de *in vivo* H^+/ATP stoichiometrie van deze isovormen onderzocht door de anaërobe biomassaopbrengst op maltose te meten. Een verhoogde H^+/ATP stoichiometrie moet theoretisch gezien leiden tot een verhoogde biomassaopbrengst op maltose als gevolg van een verminderde ATP-hydrolyse voor export van het met maltose geïmporteerde proton. Om de *in vivo* H^+/ATP stoichiometrie van de Ser800Ala en Glu803Gln isovormen van Pma1p te bepalen, is het natieve *PMA1*-allel vervangen door de overeenkomstige *PMA1*-mutantallelen in een *pma2*Δ stamachtergrond en zijn de anaërobe biomassaopbrengsten vergeleken met een isogene *PMA1 pma2*Δ referentiestam. Er werden hierbij echter geen verschillen in biomassaopbrengst waargenomen zodat geconcludeerd kan worden dat introductie van deze mutaties in *PMA1* de *in vivo* H^+/ATP stoichiometrie niet verhoogt en dat een verhoogde *in vitro* H^+/ATP stoichiometrie geen verhoogde *in vivo* stoichiometrie garandeert. De in deze studie ontwikkelde methode is een nuttig instrument in de voortdurende zoektocht naar plasmamembraan H^+-ATPase-isovormen met een *in vivo* H^+/ATP- stoichiometrie groter dan één.

ATP-neutrale productvormingsroutes, zoals melkzuur- en appelzuurproductie in gemodificeerde *S. cerevisiae* stammen, kunnen profiteren van de strategieën om vrije-energie (ATP) conservering te verhogen, zoals onderzocht in hoofdstukken 3 en 4. Daarentegen kunnen industriële processen, zoals bioethanolproductie, waarin productvorming resulteert in een netto productie van ATP, juist profiteren van verminderde energieconservering, aangezien een surplus aan ATP resulteert in overtollige biomassavorming, wat weer ten koste gaat van productvorming. In zulke gevallen is het verlagen van de ATP-opbrengst een interessante strategie om de productopbrengst te verhogen. In **hoofdstuk 5** is dit concept onderzocht voor de alcoholische vergisting van sucrose, de grondstof voor ongeveer de helft van de wereldwijde productie van bioethanol. In *S. cerevisiae* wordt de disaccharide sucrose voornamelijk buiten de cel gehydrolyseerd waarna de gevormde monosaccharides via gefaciliteerde diffusie worden opgenomen. De daaropvolgende omzetting naar ethanol via de Embden-

Meyerhof-Parnas glycolytische stofwisselingsroute levert 4 ATP per sucrose op. *S. cerevisiae* kan sucrose echter ook importeren via een sucrose-proton-symport mechanisme en vervolgens intracellulair hydrolyseren naar glucose en fructose. Uitscheiding van het geïmporteerde proton door het plasmamembraan H^+-ATPase kost 1 ATP en daarom levert intracellulaire sucrosehydrolyse slechts 3 ATP per sucrose op. Als gevolg van de ATP-behoefte voor sucrose-import, leidt intracellulaire hydrolyse van sucrose theoretisch tot een daling van de biomassaopbrengst met 25% en een verhoging van de ethanolopbrengst met 9%. Deze hypothese is getest door de promotor en 5'-coderende sequenties van het invertase-gen *SUC2* aan te passen, hetgeen resulteerde in een voornamelijk (94%) cytosolische lokalisatie van sucrosehydrolyse. In anaërobe sucrose-gelimiteerde chemostaten vertoonde deze gemodificeerde i*SUC2*-stam echter slechts een 4% verhoogde ethanolopbrengst. Daarnaast gaf een verhoogde residuele sucrose concentratie (circa 2 $g \cdot l^{-1}$) aanwijzingen voor een suboptimale sucrose-transportkinetiek. Om de sucrose-opnameaffiniteit te verbeteren, is deze i*SUC2*-stam onderworpen aan 90 generaties van laboratoriumevolutie in anaërobe sucrose-gelimiteerde chemostaten. Deze evolutie resulteerde in een twintigvoudige daling van de residuele sucrose concentratie en een tienvoudige toename van de sucrose-transportcapaciteit. Een geïsoleerde kolonie van deze evolutiecultuur vertoonde een 11% hogere ethanolopbrengst en een 30% lagere biomassopbrengst in chemostaatculturen in vergelijking met een isogene *SUC2* referentiestam. Transcriptoomanalyse van deze geëvolueerde giststam onthulde een verhoogde expressie van *AGT1*, een gen dat codeert voor een disaccharide-proton symporter, en andere maltose-gerelateerde genen. Deletiestudies en diagnostische PCR toonden aan dat het *AGT1*-gen was gedupliceerd tijdens het evolutie-experiment. Na deletie van beide kopieën van *AGT1* keerde de residuele suikerconcentratie en transportcapaciteit terug naar die van de niet-geëvolueerde i*SUC2*-stam en de biomassa- en ethanolopbrengst terug naar die van een *SUC2*-referentiestam. Het verhuizen van sucrosehydrolyse om de ethanolopbrengst te verhogen, is toegepast in industriële stamachtergronden en de prestaties van de resulterende stammen onder industriële omstandigheden worden onderzocht.

Het onderzoek beschreven in dit proefschrift illustreert dat de efficiëntie van vrije-energie (ATP) conservering van belang is voor efficiënte productie van verbindingen met een laag moleculair gewicht, zoals melkzuur en ethanol. Voor productvormingsroutes die leiden tot een overmaat aan ATP en daarmee tot

ongewenste biomassaproductie, is een breed toepasbare strategie (verhuizing van sucrosehydrolyse) gepresenteerd om de conservering van vrije energie (ATP) te verlagen en zo productvorming te verbeteren. Andersom kan de efficiëntie van vrije-energieconservering uit de maltosestofwisseling worden verhoogd door expressie van een maltosefosforylase. Deze aanpak vormt de basis voor verdere ontwikkeling van strategieën die erop gericht zijn de ATP-opbrengst van industrieel relevante productvormingsroutes te verhogen, om zo uiteindelijk processen als anaërobe melkzuurproductie met *S. cerevisiae* mogelijk te maken.

Curriculum Vitae - English

Stefan de Kok was born on June 24th 1983 in Burgerveen, the Netherlands, where he was also raised. After attending gymnasium at the Herbert Vissers College in Nieuw-Vennep and graduating in 2001, Stefan studied Life Science & Technology at Leiden University and Delft University of Technology (TU Delft). In 2005, he obtained his Bachelor of Science diploma (with honours) by completing a research project on xylose-fermenting *Saccharomyces cerevisiae* strains in the Industrial Microbiology Section (TU Delft) under the supervision of Marko Kuyper and Jack Pronk. In 2006, he performed an industrial internship at The BioAffinity Company, working on the industrial production of monoclonal antibodies with *S. cerevisiae* under the supervision of Teun van der Laar. In 2007, he finished a research project on the energetics of hydrogen production in mixed culture fermentations in the Environmental Biotechnology Section (TU Delft) under the supervision of Jasper Meijer, Robbert Kleerebezem and Mark van Loosdrecht and thereby obtained his Master of Science diploma (with honours). In September 2007, Stefan started a PhD project on 'metabolic engineering of free-energy (ATP) conserving reactions in *S. cerevisiae*' in the Industrial Microbiology Section (TU Delft) under the supervision of Ton van Maris and Jack Pronk. Currently, Stefan is working as industrial postdoctoral researcher for Amyris in Emeryville, California, USA.

Curriculum Vitae - Nederlands

Stefan de Kok werd geboren op 24 juni 1983 in Burgerveen, waar hij ook is opgegroeid. Hij heeft het gymnasium gevolgd op het Herbert Vissers College in Nieuw-Vennep en met succes afgerond in 2001. Daarna is hij Life Science & Technology gaan studeren aan de Universiteit van Leiden en de Technische Universiteit Delft. In 2005 heeft hij zijn Bachelor of Science diploma (met lof) behaald door het afronden van een onderzoeksproject over xylose fermenterende *Saccharomyces cerevisiae* stammen in de Industriële Microbiologie sectie (TU Delft) onder begeleiding van Marko Kuyper en Jack Pronk. In 2006 heeft Stefan een bedrijfsstage verricht bij The BioAffinity Company, waar hij heeft gewerkt aan de industriële productie van monoklonale antilichamen met *S. cerevisiae* onder begeleiding van Teun van der Laar. In 2007 heeft hij een onderzoeksproject afgerond over de energetica van waterstofproductie in mengpopulatie-fermentaties in de Milieubiotechnologie sectie (TU Delft) onder de begeleiding van Jasper Meijer, Robbert Kleerebezem en Mark van Loosdrecht en daarmee zijn Master of Science diploma (met lof) behaald. In september 2007 is Stefan een promotieproject begonnen met als onderwerp 'metabolic engineering van vrije-energie (ATP) conserverende reacties in *Saccharomyces cerevisiae*' in de Industriële Microbiologie sectie (TU Delft) onder de begeleiding van Ton van Maris en Jack Pronk. Momenteel werkt Stefan als postdoctorale onderzoeker bij Amyris in Emeryville, Californië, Verenigde Staten.

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