Inactivation of the *Kluyveromyces lactis* *KIPDA1* gene leads to loss of pyruvate dehydrogenase activity, impairs growth on glucose and triggers aerobic alcoholic fermentation

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The *KIPDA1* gene, encoding the E1 α subunit of the mitochondrial pyruvate-dehydrogenase (PDH) complex was isolated from a *Kluyveromyces lactis* genomic library by screening with a 1.1 kb internal fragment of the *Saccharomyces cerevisiae* *PDA1* gene. The predicted amino acid sequence encoded by *KIPDA1* showed 87% similarity and 79% identity to its *S. cerevisiae* counterpart. Disruption of *KIPDA1* resulted in complete absence of PDH activity in cell extracts. The maximum specific growth rate on glucose of null mutants was 3.5-fold lower than that of the wild-type, whereas growth on ethanol was unaffected. Wild-type *K. lactis* CBS 2359 exhibits a Crabtree-negative phenotype, i.e. no ethanol was produced in aerobic batch cultures grown on glucose. In contrast, substantial amounts of ethanol and acetaldehyde were produced in aerobic cultures of an isogenic *Klida1* null mutant. A wild-type specific growth rate was restored after introduction of an intact *KIPDA1* gene but not, as previously found for *S. cerevisiae* *pda1* mutants, by cultivation in the presence of leucine. The occurrence of aerobic fermentation and slow growth of the *Klida1* null mutant indicate that, although present, the enzymes of the PDH bypass (pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase) could not efficiently replace the PDH complex during batch cultivation on glucose. Only at relatively low growth rates ($D = 0.10\text{ h}^{-1}$) in aerobic, glucose-limited chemostat cultures, could the PDH bypass completely replace the PDH complex, thus allowing fully respiratory growth. This resulted in a lower biomass yield [g biomass (g glucose)⁻¹] than in the wild-type due to a higher consumption of ATP in the PDH bypass compared to the formation of acetyl-CoA via the PDH complex.

Keywords: *Kluyveromyces lactis*, pyruvate dehydrogenase, Crabtree effect, aerobic fermentation, yeast

INTRODUCTION

Pyruvate is a key intermediate in sugar dissimilation by yeasts which, depending on growth conditions, can either be converted into acetyl-CoA, the fuel of the TCA

cycle, or be decarboxylated and reduced to ethanol (Fig. 1). The mode of pyruvate dissimilation affects the biomass yield on sugar, as the ATP yield from respiratory dissimilation via the TCA cycle is much higher than that from alcoholic fermentation (Verduyn, 1991).

Some yeasts, including *Saccharomyces cerevisiae*, exhibit alcoholic fermentation even during fully aerobic growth on sugars in batch cultures. This phenomenon, known as the Crabtree effect (de Deken, 1966), involves

Abbreviation: PDH, pyruvate dehydrogenase.

The EMBL accession number for the sequence reported in this paper is AF023920.

Fig. 1. Enzymes of pyruvate dissimilation. Numbered reactions are catalysed by the following enzymes: 1, pyruvate dehydrogenase complex; 2, pyruvate decarboxylase; 3, acetaldehyde dehydrogenase; 4, acetyl-CoA synthetase; 5, alcohol dehydrogenase.

repression of the synthesis of respiratory enzymes by glucose (Fiechter *et al.*, 1981; van Dijken & Scheffers, 1986). In Crabtree-positive yeasts, alcoholic fermentation can be avoided by aerobic, sugar-limited cultivation at low specific growth rates (Fiechter *et al.*, 1981). This is a major complication in biomass-directed industrial applications (e.g. heterologous-protein production; Hensing *et al.*, 1995). Even during aerobic, sugar-limited growth, exposure of respiring Crabtree-positive yeasts to excess glucose triggers instantaneous ethanol production. This rapid response, which does not involve regulation at the level of enzyme synthesis, has been interpreted in terms of a limited capacity of respiratory sugar metabolism, leading to overflow of glycolysis into ethanol production (Käppeli, 1986; Sonnleitner, 1991). This limited respiratory capacity has not yet been attributed to any specific biochemical reaction(s) (Pronk *et al.*, 1996).

The genetics and physiology of pyruvate metabolism have been extensively studied in *S. cerevisiae* (for reviews see Pronk *et al.*, 1996; Hohmann, 1997; Steensma, 1997), but there has been little research on the Crabtree-negative yeasts. Still, knowledge of the differences in pyruvate metabolism between *S. cerevisiae* and Crabtree-negative yeasts is relevant, as this might guide genetic-engineering strategies to eliminate aerobic fermentation in Crabtree-positive yeasts.

Kluyveromyces lactis is a popular model organism for molecular studies on the regulation of sugar metabolism and respiration (Węsółowski-Louvel *et al.*, 1996). Research on *K. lactis* also has industrial relevance, as it is used for the production of β -galactosidase and heterologous proteins (Swinkels *et al.*, 1993). Since, moreover, this yeast does not produce ethanol under a variety of aerobic growth conditions (Kiers *et al.*, 1998), *K. lactis* is

a suitable Crabtree-negative model yeast for studies on pyruvate metabolism.

In yeasts, the mitochondrial pyruvate dehydrogenase (PDH) complex is a large, strongly conserved multi-enzyme complex, catalysing the oxidative decarboxylation of pyruvate to acetyl-CoA (overall reaction: $\text{pyruvate} + \text{NAD}^+ + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH}$; Fig. 1). Pyruvate is first linked to thiamin pyrophosphate (TPP), the cofactor of the E1 subunit of PDH (EC 1.2.4.1). The product of the E1 reaction, 2- α -hydroxy-ethyl-TPP, is then oxidized and coupled to the lipoamide cofactor of the E2 subunit from where it is finally transferred to CoA. The lipoamide group of E2, the electron acceptor for oxidation of 2- α -hydroxy-ethyl-TPP, is regenerated via reduction of NAD^+ by the E3 subunit. The same E3 subunit occurs in PDH, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase; the E1 and E2 subunits are complex specific (Reed, 1974).

In *S. cerevisiae*, as in most other organisms, the E1 subunit of the PDH complex consists of two proteins, E1 α and E1 β (Kresze & Ronft, 1981). The *PDA1* gene encoding the E1 α subunit of the *S. cerevisiae* PDH complex has been cloned (Behal *et al.*, 1989; Steensma *et al.*, 1990) and the physiological consequences of null mutations have been studied in detail (Wenzel *et al.*, 1992a; Pronk *et al.*, 1994). Although these mutants completely lacked PDH activity, conversion of pyruvate into acetyl-CoA still occurred via an alternative route, the PDH bypass. This bypass catalyses the conversion of pyruvate into acetyl-CoA by the concerted action of pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase (Fig. 1). Nevertheless, *pda1* null mutants exhibited some clear phenotypes. In aerobic batch cultures, growth rates in glucose were substantially lower than those of the wild-type, but could be almost completely restored by addition of leucine. This was tentatively explained as a regulatory effect of a changed intra-mitochondrial acetyl-CoA/CoA ratio on α -isopropylmalate synthase, a key enzyme in leucine biosynthesis (Wenzel *et al.*, 1992a). Furthermore, *pda1* mutants exhibited a lower biomass yield on glucose in aerobic, glucose-limited chemostat cultures, although growth was completely respiratory. This was explained by ATP consumption in the PDH bypass (Pronk *et al.*, 1994).

The phenotype of *K. lactis* mutants lacking pyruvate decarboxylase, the key enzyme of fermentative pyruvate metabolism, differs strongly from that of corresponding *S. cerevisiae* mutants. *S. cerevisiae* *pdc* null mutants cannot grow on glucose as the sole carbon source (Flikweert *et al.*, 1996), whereas null mutants in the single *KLPDCA* gene of *K. lactis* grow normally on glucose (Bianchi *et al.*, 1996). At present, nothing is known about the genetics of the PDH complex in *K. lactis*. The aims of the present study were to construct a *K. lactis* null mutant lacking PDH activity and to compare its phenotype with an isogenic wild-type strain. Special attention was paid to the occurrence of alcoholic

aerobic fermentation in glucose-grown batch and chemostat cultures.

METHODS

Yeast strains and media. The yeast strains used in this study are listed in Table 1. YPD medium, used for maintenance of yeast strains and recombinant-DNA procedures, contained (per litre): yeast extract, 10 g; Bacto peptone, 20 g; glucose, 10 g. *Escherichia coli* strains containing plasmids were grown in LB medium (per litre: yeast extract, 5 g; Bacto tryptone, 10 g; NaCl, 10 g; pH 7.0) or TB medium (per litre: Bacto tryptone, 12 g; yeast extract, 24 g; glycerol, 4 g; KH_2PO_4 , 2.31 g; K_2HPO_4 , 12.54 g). Ampicillin was used at a concentration of 60 mg l^{-1} . For physiological characterization of *K. lactis* strains, a defined mineral medium supplemented with vitamins (Verduyn *et al.*, 1992) was used. The concentration of nicotinic acid in this medium was increased to 5 mg l^{-1} (Kiers *et al.*, 1998). Carbon sources were added at the concentrations indicated. Where indicated, L-leucine was added at 0.4 g l^{-1} .

Recombinant DNA techniques. Standard protocols were followed for plasmid isolations, restriction enzyme analyses, PCR amplifications, ligations, gel electrophoresis and hybridizations (Sambrook *et al.*, 1989). Yeast chromosomal DNA was isolated according to Holm *et al.* (1986). For pulsed-field gel electrophoresis in a contour-clamped homogeneous electric field (CHEF) apparatus, chromosomal DNA was prepared as described by Teunissen *et al.* (1993). Electrophoresis was performed in 0.6% agarose (Seakem, FMC Bioproducts) in 0.5× TBE (Sambrook *et al.*, 1989) for 70 h at 100 V, with the pulse time varying linearly between 100 and 500 s. Probes were radioactively labelled by using [^{32}P]dCTP and a commercial oligonucleotide labelling kit (Pharmacia). Standard procedures were used for hybridization and washing (Sambrook *et al.*, 1989).

Cloning and sequencing of the KIPDA1 gene. The *KIPDA1* gene was cloned by low-stringency hybridization of a *K. lactis* genomic library constructed by ligation of *Sau*3A partially digested chromosomal DNA into the *Bam*HI-digested vector YRP14 ∇ SUPKARS (Heus *et al.*, 1990). Colonies were transferred to nitrocellulose membranes and treated according to the method of Woods (1984) to expose the plasmid DNA. Hybridizations were performed at 50 °C with a ^{32}P -labelled PCR probe containing 1.1 kb of the 1.3 kb *S. cerevisiae* PDA1 ORF (primers: 5' GGTCATGTTCTGACCAT 3' and 5' AGATCAATCAAATGCAT 3').

DNA sequences were determined on an ALF DNA sequencer

(Pharmacia). The region hybridizing to the *ScPDA1* probe with flanking regions of about 1 kb on either side was sequenced. Each base was covered at least three times. Sequence data were analysed with Gene skipper (EMBL). The GenEMBL (release 86.0) and SWISS-PROT (release 30.0) databases were screened using the BLAST program (Altschul *et al.*, 1990) to identify DNA and protein similarities, respectively.

Disruption of the KIPDA1 gene. The *KIPDA1* gene was disrupted by replacing the 100 bp internal *Kpn*I–*Hind*III fragment with a 1.1 kb *Kpn*I–*Hind*III fragment from pUT332 containing the dominant selectable Tn5BLE marker gene under control of the *S. cerevisiae* *TEF1* promoter and *CYC1* terminator (Gatignol *et al.*, 1990). The *Sca*I–*Xba*I fragment of the construct (see Fig. 2) was used to transform *K. lactis* CBS 2359. Published procedures were followed with regard to electroporation and incubation times after transformation (Wenzel *et al.*, 1992b). The concentration of phleomycin used for selection on the YPD plates was 8 µg (ml medium) $^{-1}$.

PCR analysis of the disrupted KIPDA1 gene. Preliminary analysis of the transformants was carried out by performing PCR reactions on whole yeast cells. The reaction mixture contained: 10 mM Tris/HCl (pH 8.3); 50 mM KCl, 2.5 mM MgCl_2 , 0.17 g BSA (acetylated) l^{-1} , 0.2 mM dNTPs and approximately 1 ng primer per 50 µl reaction. The annealing temperature was 55 °C. Oligonucleotide sequences of the primers used are 5' CACTCCTCTGTCTGGTCAA 3' and 5' GAGAGCATTATGGGTGG 3'. The length of the PCR product from wild-type cells was 2.5 kb whereas PCR on the transformants with the correct integration resulted in 3.5 kb products.

Complementation of Klpda1 null mutants. Strain GG 1990 was grown on 5-fluoroorotic acid plates (Boeke *et al.*, 1984) to isolate *ura3* and *ura5* mutants. *ura3* mutants were selected by complementation of their uracil auxotrophy with the single-copy plasmid pRW336, containing the *S. cerevisiae* *URA3* gene (Winkler *et al.*, 1998). The *Klpda1::Tn5BLE*, *ura3-49* strain obtained in this way was called GG 1993. This strain was transformed with either the empty vector pRW336 (to give GG 1993 pRW) or with pRW-*KIPDA1* (to give GG 1993 PDA), which was obtained by ligating the largest *Sca*I–*Xba*I fragment of the construct, containing the complete ORF and promoter of *KIPDA1* (see Fig. 2), into *Sma*I–*Xba*I-digested pRW336.

Northern analysis. Published procedures were used for isolation of total RNA and subsequent analysis (van den Berg *et al.*, 1996). Hybridizations were performed at 65 °C with a ^{32}P -labelled PCR probe containing the *KIPDA1* ORF. The primer

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
<i>K. lactis</i>		
CBS 2359	Wild-type	Wésolowski-Louvel <i>et al.</i> (1996)
GG 1990	<i>Klpda1::Tn5BLE</i>	This study
GG 1993	<i>Klpda1::Tn5BLE ura3-49</i>	This study
GG 1993 pRW	<i>Klpda1::Tn5BLE ura3-49</i> , pRW336	This study
GG 1993 PDA	<i>Klpda1::Tn5BLE ura3-49</i> , pRW- <i>KIPDA1</i>	This study
<i>S. cerevisiae</i>		
T2-3D	Wild-type	Wenzel <i>et al.</i> (1992a)
T2-3C	<i>pda1::Tn5BLE/pda1::Tn5BLE</i>	Wenzel <i>et al.</i> (1992a)

sequences were 5' TCAGAACCTGGAACGTAG 3' and 5' GCTCAATCCTCTGTGGTT 3'. Published procedures were used for hybridization and washing (van den Berg *et al.*, 1996).

Cultivation conditions. Batch cultivations were performed at 30 °C in 2 litre Applikon fermenters with a working volume of 1.5 litres. The stirring speed was set at 1000 r.p.m. and the vessel was sparged with 1 litre air min⁻¹, administered via a Brooks 5876 mass-flow controller. The pH was maintained at pH 5.0 by automatic addition of 2 M KOH. The dissolved oxygen tension was measured continuously with an Ingold polarographic oxygen electrode. Aerobic chemostat cultivation was performed at 30 °C, pH 5.0, in the same fermenters, equipped with pH control and an oxygen electrode. The working volume was kept constant at 1 litre by a peristaltic pump coupled to an electrical level sensor. The stirring speed was set at 800 r.p.m. and the dilution rate at 0.10 h⁻¹.

Gas analysis. The exhaust gas of fermenter cultures was cooled in a condenser (2 °C) and dried with an Perma Pure dryer (PD-625-12P). Oxygen and carbon dioxide concentrations were determined with a Servomex 1100A analyser and a Beckman model 864 infrared detector, respectively. The exhaust gas flow rate was measured as described previously (Weusthuis *et al.*, 1994). Specific rates of oxygen consumption and carbon dioxide production were calculated according to van Urk *et al.* (1988).

Analytical procedures. In batch cultures, growth was monitored as OD₆₆₀ on a Vitalab spectrophotometer, with samples taken each hour. When necessary, cell suspensions were diluted to ensure that OD₆₆₀ did not exceed 0.3. In chemostat cultures, biomass dry weights were determined as described by Postma *et al.* (1989). Glucose concentrations in media and culture supernatants (obtained by centrifugation at 20000 g for 5 min) were determined colorimetrically with the GOD-PAP method (Merck systems kit 14144). Ethanol in the supernatants was determined with a colorimetric assay (Verduyn *et al.*, 1984) based on *Hansenula polymorpha* alcohol oxidase (kindly provided by BIRD Engineering, Schiedam, The Netherlands). Other metabolites were determined by HPLC as described by Flikweert *et al.* (1997).

Preparation of cell extracts. Culture samples were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, concentrated fourfold and stored at -20 °C. Cell disruption was performed either by sonication or by use of a French press. Before sonication, samples were thawed on ice, washed, and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM MgCl₂ and 1 mM DTT. Sonication was carried out at 0 °C for 2 min in 0.5 min pulses with an MSE sonicator (150 W output, 7 µm peak-to-peak amplitude). Before French press disruption, samples were thawed on ice, washed and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 0.05% (w/v) Triton X-100, 2 mM MgCl₂, 0.5 mg leupeptin l⁻¹, 0.7 mg pepstatin l⁻¹ and 2 mM PMSF. Cells were then disrupted by three passages at 4 °C, 1000 p.s.i. (12.9 MPa), through an American Instrument Company FA-073 French pressure cell. After cell disruption by either sonication or French press, unbroken cells and debris were removed by centrifugation for 20 min at 36000 g, 4 °C. Cell extracts used for PDH assays were dialysed overnight at 4 °C against 100 mM potassium phosphate (pH 7.5) containing 2 mM MgCl₂ and 1 mM DTT. This dialysis step was essential to remove low-molecular-mass metabolites that interfered with the PDH assay. Protein contents of cell extracts were determined with the Lowry method, using BSA (fatty-acid free; Sigma) as a standard.

Enzyme assays. Spectrophotometric enzyme assays were carried out with a Hitachi spectrophotometer (model 100-60) at 30 °C with freshly prepared cell extracts. All enzyme activities are expressed as µmol substrate min⁻¹ (mg protein)⁻¹, based on an absorption coefficient of reduced pyridine nucleotide cofactors at 340 nm of 6.3 mM⁻¹ cm⁻¹. Activity of the PDH complex was assayed with dialysed French-press extracts as described by Flikweert *et al.* (1997). The pH of the assay (pH 8.0) and the high concentration of phosphate prevented interference by pyruvate decarboxylase. Activities of other enzymes were determined in cell extracts prepared by sonication. Acetyl-CoA synthetase (EC 6.2.1.1) was assayed according to van den Berg *et al.* (1996); glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49), (acet)aldehyde dehydrogenases (NAD⁺ dependent and NADP⁺ dependent; EC 1.2.1.4 and EC 1.2.1.5, respectively), alcohol dehydrogenase (EC 1.1.1.1) and pyruvate decarboxylase (EC 4.1.1.1) were assayed according to Postma *et al.* (1989).

RESULTS

Cloning and disruption of the *KIPDA1* gene

The structural gene encoding the E1α subunit of the *K. lactis* PDH complex was isolated by low-stringency hybridization of a genomic library with a 1.1 kb internal PCR fragment of the *S. cerevisiae* *PDA1* gene (Steensma *et al.*, 1990). Three positive clones were obtained from 4000 colonies, which covered the *K. lactis* genome approximately four times. Restriction analysis demonstrated that the *K. lactis* inserts of the three hybridizing plasmids partially overlapped (Fig. 2). The position of the putative *K. lactis* *PDA1* gene was determined more precisely by hybridization to the *S. cerevisiae* *PDA1* fragment. The clone containing the largest insert was used for sequencing. The sequence revealed an ORF of 1.3 kb with 79% similarity to the *S. cerevisiae* gene. At the amino acid level the identity was 79% (87% similarity), with only limited similarity at the N-termini of the predicted amino acid sequences. The gene was shown to be located on chromosome V of *K. lactis* by Southern hybridization of a CHEF-blot with the *S. cerevisiae* *PDA1* probe (data not shown).

Upstream of the putative *KIPDA1* gene, a 1.0 kb ORF was identified. This unknown ORF exhibited 58% identity and 71% similarity with a putative membrane protein of unknown function on chromosome IV of *S. cerevisiae* (YDR101c; Jacq *et al.*, 1997). Downstream of the *KIPDA1* gene, similarity was found with the *S. cerevisiae* *DMC1* gene, a meiosis-specific yeast homologue of *E. coli* *recA*, which is located downstream of *ScPDA1* (Bishop *et al.*, 1992). However, no ORF was found in this region.

To test whether the cloned ORF was indeed a functional *KIPDA1* gene, a disruption was made in the wild-type *K. lactis* strain CBS 2359 using a dominant phleomycin-resistance marker (see Methods; Fig. 2). Phleomycin-resistant colonies were tested for correct integration of the marker gene at the *PDA1* locus. The results confirmed the low efficiency of integration by homologous recombination in *K. lactis* in comparison to *S. cerevisiae* (Rossolini *et al.*, 1992). In an initial attempt,

Fig. 2. Clones containing fragments hybridizing to the *S. cerevisiae* PDA1 probe. The 4.8 kb region between the *Hind*III site in the vector and the *Bam*HI site in the insert of clone 5 was sequenced. Disruption of the *KIPDA1* gene was obtained by replacing a 100 bp *Hind*III–*Kpn*I fragment with the dominant *Tn5BLE* marker on a *Hind*III–*Kpn*I fragment. The *Scal*–*Xba*I fragment was used for transformation. Double crossovers were selected by resistance to phleomycin and analysed further. B, *Bam*HI; Bg, *Bgl*II; E1, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Scal*; Sa, *Sall*; X, *Xba*I.

40 phleomycin-resistant transformants from both haploid and diploid strains were analysed by Southern blotting and were all found to contain non-homologous integrations. In a further attempt, using PCR as a first screen, 14 of 20 phleomycin-resistant colonies of CBS 2359 (and 9 of 116 other wild-type strains transformed with the disruption fragment) lacked the PCR product representing an intact *KIPDA1* locus. All but two of these showed the expected hybridizing DNA fragments for correct integration at the *KIPDA1* locus on Southern blots using three different restriction enzymes (data not shown). In Northern analyses, the eight disruptants tested all yielded a transcript of 1.5 kb, which is smaller than the 2.8 kb message from the intact gene. This indicates that the 5' part of the ORF remaining in the disruptant (Fig. 2) was still transcribed.

One of the disruption mutants constructed in *K. lactis* CBS 2359, named GG 1990, was chosen for further characterization. PDH activity was below the detection limit of 5 nmol substrate min⁻¹ (mg protein)⁻¹ in cell extracts of this strain grown in batch cultures on glucose. Conversely, significant enzyme activity [75 ± 10 nmol substrate min⁻¹ (mg protein)⁻¹] was detected in corresponding extracts of the isogenic wild-type.

Growth in shake-flask cultures

For an initial physiological characterization of the PDH-negative strain *K. lactis* GG 1990, its growth in shake-flask cultures was compared with that of the isogenic wild-type strain, *K. lactis* CBS 2359. The wild-type and mutant showed the same specific growth rates in defined media containing ethanol as the sole carbon source (Table 2). However, growth on glucose was severely impaired in the *Klpda1* disruption mutant, its specific growth rate being 3.5-fold lower than that of the wild-type strain. Growth was restored to wild-type levels by introduction of the intact gene on a single-copy vector (Table 2).

In *S. cerevisiae*, the reduced specific growth rate of *pda1* null mutants on glucose can be partly restored by

addition of leucine (Wenzel *et al.*, 1992a). However, addition of 0.4 g leucine l⁻¹ did not have any effect on the specific growth rate on glucose of either the *Klpda1* null mutant or wild-type *K. lactis* (Table 2). A control experiment confirmed that this concentration of leucine restored the growth rate of an *S. cerevisiae* *pda1* null mutant to 75% of the wild-type value (Table 2), consistent with the original observations of Wenzel *et al.* (1992a).

Aerobic growth in fermenter cultures

Although shake-flask experiments provided a first indication of the effect of the *Klpda1* disruption on the maximum specific growth rate, this cultivation system is poorly suited for a more detailed characterization of growth and product formation. It is well known that shake-flask cultures readily become oxygen limited due to poor oxygen transfer. This in itself is sufficient to trigger alcoholic fermentation in glucose-grown *K. lactis* cultures (Kiers *et al.*, 1998), as well as in cultures of other yeasts (van Dijken & Scheffers, 1986). To circumvent this problem, growth of the two strains on glucose was studied in aerobic fermenter cultures (see Methods).

The specific growth rates in the fermenter cultures, which were operated at a dissolved oxygen concentration above 50% of air saturation, confirmed the negative effect of the *Klpda1* null mutation observed in shake flasks (Fig. 3, Table 2). The maximum growth rates on glucose found in fermenters and shake flasks were almost the same (Table 2). However, the exponential growth phase in the fermenter lasted longer than in the shake flasks (data not shown). Both the better availability of oxygen and the control of culture pH in the fermenters may have contributed to this prolonged exponential growth. As expected from the lower growth rate, glucose consumption in the *Klpda1* mutant was slower than in the isogenic wild-type strain (Fig. 3).

Ethanol accumulation did not occur in the wild-type strain, confirming the Crabtree-negative phenotype of *K. lactis* CBS 2359 (Kiers *et al.*, 1998). In cultures of the

Table 2. Specific growth rates of wild-type and *pda1*-null strains of *K. lactis* and *S. cerevisiae*.

Growth rates were determined in shake-flask cultures as well as in aerobic fermenter cultures in mineral medium supplemented with various carbon sources. Initial concentrations of sugars were 10 g l^{-1} and initial concentrations of ethanol were 0.5% (v/v). Where indicated, leucine was added to a concentration of 0.4 g l^{-1} .

Strain	Relevant genotype	Mode of cultivation	Carbon source	Specific growth rate (h^{-1})
<i>K. lactis</i>				
CBS 2359	<i>KIPDA1</i>	Shake flask	Ethanol	0.29
GG 1990	<i>Klpda1::Tn5BLE</i>	Shake flask	Ethanol	0.29
CBS 2359	<i>KIPDA1</i>	Shake flask	Glucose	0.41
CBS 2359	<i>KIPDA1</i>	Fermenter	Glucose	0.39
GG 1990	<i>Klpda1::Tn5BLE</i>	Shake flask	Glucose	0.12
GG 1990	<i>Klpda1::Tn5BLE</i>	Fermenter	Glucose	0.12
GG 1990	<i>Klpda1::Tn5BLE</i>	Shake flask	Glucose + Leu	0.12
GG 1993 pRW	<i>Klpda1::Tn5BLE</i> , pRW336	Shake flask	Glucose	0.12
GG 1993 PDA	<i>Klpda1::Tn5BLE</i> , pRW336- <i>KIPDA1</i>	Shake flask	Glucose	0.38
<i>S. cerevisiae</i>				
T2-3D	<i>PDA1/PDA1</i>	Shake flask	Glucose	0.45
T2-3C	<i>pda1::Tn5BLE/pda1::Tn5BLE</i>	Shake flask	Glucose	0.20
T2-3C	<i>pda1::Tn5BLE/pda1::Tn5BLE</i>	Shake flask	Glucose + Leu	0.34

Fig. 3. Growth of wild-type *K. lactis* CBS 2359 (a) and the isogenic *Klpda1* null mutant GG 1990 (b) in aerobic batch fermenter cultures (pH 5.0, 30 °C) on a defined mineral medium containing 10 g l^{-1} as the sole carbon source. During cultivation, the dissolved oxygen concentration remained above 50% of air saturation. ●, Glucose concentration; ○, OD_{660} ; □, ethanol concentration.

mutant, however, ethanol concentrations of up to 20 mM were detectable (Fig. 3) despite the fact that the dissolved oxygen concentration in the cultures did not decrease below 50% of air saturation. In addition to ethanol, acetaldehyde was produced by the *Klpda1* mutant, but was absent from wild-type cultures. Concentrations of acetaldehyde in culture supernatants of the *Klpda1* mutant were in the low millimolar range (data not shown). In view of its high volatility, most of the acetaldehyde produced was probably lost in the off gas,

where its characteristic smell was clearly detectable. Concentrations of pyruvate and acetate remained below 1 mM in both wild-type and mutant cultures.

Growth in chemostat cultures

The occurrence of aerobic alcoholic fermentation in batch cultures suggests that the *Klpda1* null mutation has converted *K. lactis* into a Crabtree-positive yeast. However, in Crabtree-positive yeast species, aerobic

Table 3. Growth of *K. lactis* CBS 2359 (wild-type) and GG 1990 (isogenic *Klpda1* null mutant) in aerobic, carbon- and energy-limited chemostat cultures ($D = 0.10 \text{ h}^{-1}$, pH 5, 30°C).

The concentration of carbon substrate in the reservoir medium was 0.25 mol l^{-1} ; residual substrate concentrations were below 1 mmol l^{-1} . In all steady-state cultures, over 95 % of the ingoing carbon could be recovered as biomass and carbon dioxide (not shown).

Strain	Substrate	Biomass yield [g dry wt (g substrate) $^{-1}$]	q_{O_2} (mmol $\text{g}^{-1} \text{ h}^{-1}$)	q_{CO_2} (mmol $\text{g}^{-1} \text{ h}^{-1}$)	RQ
CBS 2359	Ethanol	0.59 ± 0.01	3.1 ± 0.1	6.6 ± 0.1	0.5
GG 1990	Ethanol	0.58 ± 0.01	3.3 ± 0.1	7.0 ± 0.1	0.5
CBS 2359	Glucose	0.49 ± 0.01	3.0 ± 0.1	3.0 ± 0.1	1.0
GG 1990	Glucose	0.42 ± 0.01	4.0 ± 0.1	4.0 ± 0.1	1.0

alcoholic fermentation can be suppressed by growth at submaximal growth rates in glucose-limited, aerobic cultures in which the low residual glucose concentrations ($<0.1 \text{ mM}$) alleviate glucose catabolite repression (Fiechter *et al.*, 1981). To investigate whether respiratory growth of the *Klpda1* null mutant was possible under such conditions, its growth in aerobic, glucose-limited chemostat cultures was compared with that of the wild-type. For both strains, a specific growth rate (dilution rate) of 0.10 h^{-1} was selected, which is slightly below the specific growth rate on glucose of 0.12 h^{-1} found in batch cultures of the mutant strain (Table 2).

In glucose-limited chemostat cultures of the wild-type cells, glucose metabolism was completely respiratory and over 95 % of the glucose-carbon entering the cultures was recovered as biomass and carbon dioxide. In contrast to batch cultures on glucose (Fig. 3), glucose-limited chemostat cultures of the *Klpda1* null mutant exhibited a completely respiratory glucose metabolism. As in wild-type chemostat cultures, biomass and carbon dioxide were the only major products formed. Concentrations of ethanol and acetaldehyde in culture supernatants were below 1 mM and the respiratory coefficient (ratio of specific rates of oxygen consumption and carbon dioxide production) was close to unity (Table 3). This indicated that, in these glucose-limited cultures, the PDH bypass (Fig. 1) could entirely replace the function of the PDH complex in respiratory glucose dissimilation. Even though alcoholic fermentation did not occur, the biomass yield on glucose of the *Klpda1* mutant was approximately 15 % lower than that of the wild-type (Table 3). This is similar to the reduction of the biomass yield on glucose observed in a *pda1* null mutant of *S. cerevisiae* (Pronk *et al.*, 1994).

As the PDH complex is not known to be involved in ethanol metabolism by *K. lactis*, ethanol-limited chemostat cultures were used as a control for possible pleiotropic effects of the *Klpda1* null mutation. Biomass yields in ethanol-limited cultures of the wild-type and mutant strains were not significantly different (Table 3).

Activities of key enzymes in cell extracts

To further investigate the impaired growth of the *Klpda1* null mutant in glucose-grown cultures, activities of a number of key enzymes of pyruvate metabolism were measured. In cell extracts prepared from exponentially growing, aerobic batch cultures on glucose, the *Klpda1* mutant exhibited an approximately 2.5-fold higher level of the key fermentative enzymes pyruvate decarboxylase and alcohol dehydrogenase than the wild-type (Table 4). The combined levels of NAD^+ - and NADP^+ -dependent acetaldehyde dehydrogenase, the second enzyme of the PDH bypass, did not differ markedly between the two strains (Table 4). The activity of acetyl-CoA synthetase was much lower in the *Klpda1* mutant than in wild-type cells (Table 4). Consistent with the respiratory metabolism of the *Klpda1* mutant in aerobic, glucose-limited chemostat cultures (Table 3), cell extracts from such cultures exhibited substantial levels of the PDH-bypass enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase (Table 4). With the possible exception of NAD^+ -dependent acetaldehyde dehydrogenase, enzyme levels of wild-type and *Klpda1* chemostat cultures differed by less than twofold.

DISCUSSION

Molecular characteristics of the *KIPDA1* gene

Although the *K. lactis* and *S. cerevisiae* PDA1 genes are highly similar (87 % at the protein level), the N-terminal parts of the proteins differ. Limited similarity between N-terminal parts of *K. lactis* proteins and their *S. cerevisiae* counterparts has been reported earlier (Mulder *et al.*, 1994; Chen & Clark-Walker, 1996). Despite the apparent lack of similarity of the predicted N-terminal protein sequences of the *K. lactis* and *S. cerevisiae* E1 α subunits, they both are rich in basic and hydrophobic residues, typical of mitochondrial targeting sequences. When the 44 N-terminal amino acids were excluded from the sequence comparison, the similarity increased to 94 %, suggesting very similar structures of the E1 α subunits in the two yeasts.

Table 4. Activities of key enzymes in pyruvate metabolism in cell extracts of *K. lactis* CBS 2359 (wild-type) and GG 1990 (*Klpda1::Tn5BLE*), prepared from exponentially growing aerobic fermenter batch cultures on glucose and from aerobic glucose-limited chemostat cultures ($D = 0.10 \text{ h}^{-1}$)

Enzyme activities [$\mu\text{mol substrate min}^{-1} (\text{mg protein})^{-1}$] from batch cultures are shown as the mean $\pm$ SD of assays on two cell extracts prepared from the same culture; data from chemostat cultures are shown as the mean $\pm$ SD of assays on samples from two independent steady-state cultures. Glucose-6-phosphate dehydrogenase was included as a reference enzyme activity.

Enzyme	Batch		Chemostat	
	CBS 2359	GG 1990	CBS 2359	GG 1990
Glucose-6-phosphate dehydrogenase	1.1 $\pm$ 0.1	1.4 $\pm$ 0.1	0.89 $\pm$ 0.08	1.1 $\pm$ 0.1
Pyruvate decarboxylase	0.20 $\pm$ 0.02	0.51 $\pm$ 0.06	0.20 $\pm$ 0.03	0.35 $\pm$ 0.02
Alcohol dehydrogenase	2.1 $\pm$ 0.2	4.5 $\pm$ 0.6	3.9 $\pm$ 0.9	5.8 $\pm$ 0.4
Acetaldehyde dehydrogenase (NAD ⁺)	0.37 $\pm$ 0.04	0.28 $\pm$ 0.05	0.17 $\pm$ 0.03	0.45 $\pm$ 0.07
Acetaldehyde dehydrogenase (NADP ⁺)	0.14 $\pm$ 0.02	0.26 $\pm$ 0.05	0.10 $\pm$ 0.02	0.15 $\pm$ 0.02
Acetyl-CoA synthetase	0.22 $\pm$ 0.01	0.07 $\pm$ 0.02	0.78 $\pm$ 0.17	0.49 $\pm$ 0.08

In the PDH complexes of higher eukaryotes, phosphorylation of the E1 α subunit is an important regulatory mechanism. It has been demonstrated that the *S. cerevisiae* E1 α subunit can be phosphorylated *in vitro* (Uhlinger *et al.*, 1986; James *et al.*, 1995), although the physiological relevance of phosphorylation in this yeast remains unclear (Pronk *et al.*, 1996; Flikweert *et al.*, 1997). The amino acid sequence at the phosphorylation site of *S. cerevisiae* (Ser³¹³) exhibits strong similarity to those in bovine and porcine E1 α subunits (Uhlinger *et al.*, 1986). A similar sequence was found in the predicted amino acid sequence of the *KIPDA1* product, suggesting the possibility of phosphorylation as a regulatory mechanism. Whether phosphorylation is important and to what extent short-term regulation of the KIPDH complex is dependent on this phenomenon needs to be examined more closely.

The promoter region of the *KIPDA1* gene contains very few putative regulatory sequences. Except for *GCN4* boxes at positions -548, -236, -146 and -77, a *REB1* box at position -663 and two *TFIID* boxes at positions -799 and -956, no known regulatory sequences were found in the promoter region. The latter three promoter elements are closer to the ORF resembling YDR101c than to the *KIPDA1* gene. It is therefore more likely that these elements are involved in regulation of expression of the ORF with unknown function than of the *KIPDA1* gene.

Respiratory pyruvate dissimilation in *K. lactis*

The fully respiratory glucose metabolism in batch cultures of wild-type *K. lactis* is not due to absence of key enzymes of alcoholic fermentation: significant levels of pyruvate decarboxylase and alcohol dehydrogenase are present under these conditions (Table 4, Kiers *et al.*, 1998). Apparently, in wild-type *K. lactis*, respiration can compete effectively with fermentation for pyruvate and NADH. The observation that a null mutation in

KIPDA1 resulted in the occurrence of aerobic alcoholic fermentation in batch cultures (i.e. converted *K. lactis* into a Crabtree-positive yeast) indicates that the PDH complex plays a pivotal role in this respect.

In the present study, sugar metabolism in wild-type and *Klpda1* *K. lactis* was investigated in exponentially growing batch cultures and steady-state chemostat cultures. Even in wild-type *K. lactis*, the short-term response of respiring cultures to excess glucose is still incompletely documented (Kiers *et al.*, 1998). The *Klpda1* mutant presents an interesting system for further studies on this important aspect of the regulation of alcoholic fermentation in *K. lactis*.

In the absence of a functional PDH complex, decarboxylation of pyruvate to acetaldehyde by pyruvate decarboxylase does not necessarily commit pyruvate to alcoholic fermentation. A fully respiratory glucose metabolism is theoretically possible in the absence of a functional PDH complex, provided that the PDH-bypass enzymes are present at sufficient levels to channel pyruvate into the TCA cycle (Fig. 1). This situation occurred in glucose-limited chemostat cultures of the *Klpda1* null mutant which, at a dilution rate of 0.10 h^{-1} , did not exhibit aerobic fermentation (Table 3). Use of the PDH bypass is energetically less efficient than oxidative decarboxylation of pyruvate by the PDH complex, as the acetyl-CoA synthetase reaction involves hydrolysis of ATP to AMP (Fig. 1). Indeed, the biomass yield of the *Klpda1* mutant on glucose was approximately 15% lower than that of the wild-type (Table 3). Based on theoretical calculations on the rate of acetyl-CoA synthesis in growing cells and on the assumption that the PDH complex is predominantly responsible for conversion of pyruvate to acetyl-CoA in wild-type cultures, a similar 15% decrease of the biomass yield of an *S. cerevisiae* *pda1* mutant could be adequately explained from an increased ATP requirement for acetyl-CoA synthesis (Pronk *et al.*, 1994). Similar calcu-

lations on the growth energetics of the *Klpda1* mutant would require detailed data on biomass composition and respiratory energy coupling of *K. lactis*, which are currently not available. Nevertheless, it seems plausible that the same mechanism causes the low biomass yield of this strain.

The slow growth and occurrence of aerobic fermentation in batch cultures of the *Klpda1* mutant (Table 2, Fig. 3) indicate that, under these conditions, the capacity of the PDH bypass was too low to sustain respiratory growth at wild-type rates. The low acetyl-CoA synthetase activity in cell extracts prepared from batch cultures of the *Klpda1* mutant (Table 4) suggests that this enzyme might perhaps limit the *in vivo* capacity of the PDH bypass. However, enzyme activity in cell extracts does not necessarily give an accurate reflection of *in vivo* capacity. In addition to the capacity of the PDH-bypass enzymes, their subcellular localization may also be important. If acetyl-CoA synthetase is a cytosolic enzyme in *K. lactis* (as has been reported for *S. cerevisiae*; Kispal *et al.*, 1991) this would imply that dissimilation of pyruvate via the PDH bypass requires transport of acetyl-CoA into the mitochondria, probably via a carnitine-transferase system (Kohlhaw & Tan-Wilson, 1977). Further research into the regulation and subcellular compartmentation of PDH-bypass enzymes in *K. lactis* is required to identify which reaction(s) limit the *in vivo* capacity of this pathway in batch cultures of the *Klpda1* mutant.

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REFERENCES

- Altschul, S. F., Gish W., Miller, W., Myers E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Behal, R. H., Browning, K. S. & Reed, L. J. (1989). Nucleotide and deduced amino acid sequence of the alpha subunit of yeast pyruvate dehydrogenase. *Biochem Biophys Res Commun* **164**, 941–946.
- van den Berg, M. A., de Jong-Gubbels, P., Kortland, C. J., van Dijken, J. P., Pronk, J. T. & Steensma, H. Y. (1996). The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. *J Biol Chem* **271**, 28953–28959.
- Bianchi, M. M., Tizziani, L., Destruelle, M., Frontali, L. & Wésolowski-Louvel, M. (1996). The 'petite-negative' yeast *Kluyveromyces lactis* has a single gene expressing pyruvate decarboxylase activity. *Mol Microbiol* **19**, 27–36.
- Bishop, D. K., Park, D., Xu, L. & Kleckner, N. (1992). *DMC1*: a meiosis-specific homolog of *E. coli* recA required for recombination, synaptonemal complex formation, and cell cycle progression. *Cell* **69**, 439–456.
- Boeke, J. D., LaCroute, F. & Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5' phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* **197**, 345–346.
- Chen, X. J. & Clark-Walker, G. D. (1996). The mitochondrial genome integrity gene, *MGI1*, of *Kluyveromyces lactis* encodes the β -subunit of F_1 -ATPase. *Genetics* **144**, 1445–1454.
- de Deken, R. H. (1966). The Crabtree effect: a regulatory system in yeast. *J Gen Microbiol* **44**, 149–156.
- van Dijken, J. P. & Scheffers, W. A. (1986). Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol Rev* **32**, 199–224.
- Fiechter, A., Fuhrmann, G. F. & Käppeli, O. (1981). Regulation of glucose metabolism in growing yeast cells. *Adv Microb Physiol* **22**, 123–183.
- Flikweert, M. T., van der Zanden, L., Janssen, W. M. Th., van Dijken, J. P. & Pronk, J. T. (1996). Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. *Yeast* **12**, 247–257.
- Flikweert, M. T., van Dijken, J. P. & Pronk, J. T. (1997). Metabolic responses of pyruvate decarboxylase-negative *Saccharomyces cerevisiae* to glucose excess. *Appl Environ Microbiol* **63**, 3399–3404.
- Gatignol, A., Dassain, M. & Tirabi, G. (1990). Cloning of *Saccharomyces cerevisiae* promoters using a probe vector based on phleomycin resistance. *Gene* **9**, 35–41.
- Hensing, M. C. M., Rouwenhorst, R. J., Heijnen, J. J., van Dijken, J. P. & Pronk, J. T. (1995). Physiological and technological aspects of large-scale heterologous-protein production with yeasts. *Antonie Leeuwenhoek* **67**, 261–279.
- Heus, J. J., Zonneveld, B. J. M., Steensma, H. Y. & van den Berg, J. A. (1990). Centromeric DNA of *Kluyveromyces lactis*. *Curr Genet* **18**, 517–522.
- Hohmann, S. (1997). Pyruvate decarboxylases. In *Yeast Sugar Metabolism: Biochemistry, Genetics, Biotechnology and Applications*, pp. 187–211. Edited by F. K. Zimmermann & K. D. Entian. Basel: Technomic.
- Holm, C., Meeks-Wagner, C. W., Fangman, W. L. & Botstein, P. (1986). A rapid, efficient method for isolation of DNA from yeast. *Gene* **42**, 169–173.
- Jacq, C., Alt-Morbe, J., Andre, B. & 133 other authors (1997). The nucleotide sequence of *Saccharomyces cerevisiae* chromosome IV. *Nature* **387**, 75–78.
- James, A. G., Cook, R. M., West, S. M. & Lindsay, J. G. (1995). The pyruvate dehydrogenase complex of *Saccharomyces cerevisiae* is regulated by phosphorylation. *FEBS Lett* **373**, 111–114.
- Käppeli, O. (1986). Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. *Adv Microb Physiol* **28**, 181–209.
- Kiers, J., Zeeman, A. M., Luttik, M. A. H., Thiele, C., Castrillo, J. I., Steensma, H. Y., van Dijken, J. P. & Pronk, J. T. (1998). Regulation of alcoholic fermentation in batch and chemostat cultures of *Kluyveromyces lactis* CBS 2359. *Yeast* **14**, 459–469.
- Kispal, G., Cseko, J., Alkonyi, I. & Sandor, A. (1991). Isolation and characterization of carnitine acetyl-transferase from *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1085**, 217–222.
- Kohlhaw, G. B. & Tan-Wilson, A. (1977). Carnitine-acetyl-

- transferase: candidate for the transfer of acetyl groups through the mitochondrial membrane of yeast. *J Bacteriol* **129**, 1159–1161.
- Kresze, G. B. & Ronft, H. (1981). Pyruvate dehydrogenase complex from baker's yeast. *Eur J Biochem* **119**, 573–579.
- Mulder, W., Winkler, A. A., Scholten, I. H. J. M., Zonneveld, B. J. M., de Winde, J. H., Steensma, H. Y. & Grivell, L. A. (1994). Centromere promoter factors (CPF1) of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* are functionally interchangeable, despite low overall homology. *Curr Genet* **26**, 198–207.
- Postma, E., Scheffers, W. A. & van Dijken, J. P. (1989). Kinetics of growth and glucose transport in glucose limited chemostat cultures of *Saccharomyces cerevisiae* CBS8066. *Yeast* **5**, 159–165.
- Pronk, J. T., Wenzel, T. J., Luttik, M. A. H., Klaassen, C. C. M., Scheffers, W. A., Steensma, H. Y. & van Dijken, J. P. (1994). Energetic aspects of glucose metabolism in a pyruvate-dehydrogenase-negative mutant of *Saccharomyces cerevisiae*. *Microbiology* **140**, 601–610.
- Pronk, J. T., Steensma, H. Y. & van Dijken, J. P. (1996). Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12**, 1607–1633.
- Reed, L. J. (1974). Multienzyme complexes. *Accounts Chem Res* **7**, 40–46.
- Rossolini, G. M., Riccio, M. L., Gallo, E. & Galeotti, C. L. (1992). *Kluyveromyces lactis* rDNA as a target for multiple integration by homologous recombination. *Gene* **119**, 75–81.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sonnleitner, B. (1991). Dynamics of yeast metabolism and regulation. *Bioprocess Eng* **6**, 187–193.
- Steensma, H. Y. (1997). From pyruvate to acetyl-coenzyme A and oxaloacetate. In *Yeast Sugar Metabolism: Biochemistry, Genetics, Biotechnology and Applications*, pp. 339–358. Edited by F. K. Zimmermann & K. D. Entian. Basel: Technomic.
- Steensma, H. Y., Holterman, L., Dekker, I., van Sluis, C. A. & Wenzel, T. J. (1990). Molecular cloning of the gene for the E1 α subunit of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae*. *Eur J Biochem* **191**, 769–774.
- Swinkels, B. W., van Ooyen, A. J. J. & Bonekamp, F. J. (1993). The yeast *Kluyveromyces lactis* as an efficient host for heterologous protein production. *Antonie Leeuwenhoek* **64**, 187–201.
- Teunissen, A. W. R. H., van den Berg, J. A. & Steensma, H. Y. (1993). Physical localization of the flocculation gene *FLO1* on chromosome 1 of *Saccharomyces cerevisiae*. *Yeast* **9**, 1–10.
- Uhlinger, D. J., Yang, C. Y. & Reed, L. J. (1986). Phosphorylation-dephosphorylation of pyruvate dehydrogenase from yeast. *Biochemistry* **25**, 5673–5677.
- van Urk, H., Mak, P. R., Scheffers, W. A. & van Dijken, J. P. (1988). Metabolic responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon transition from glucose limitation to glucose excess. *Yeast* **4**, 283–291.
- Verduyn, C. (1991). Physiology of yeasts in relation to growth yields. *Antonie Leeuwenhoek* **60**, 325–355.
- Verduyn, C., van Dijken, J. P. & Scheffers, W. A. (1984). Colorimetric alcohol assays with alcohol oxidase. *J Microbiol Methods* **2**, 15–25.
- Verduyn, C., Postma, E., Scheffers, W. A. & van Dijken, J. P. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: a continuous culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**, 501–517.
- Wenzel, T. J., van den Berg, M. A., Visser, W., van den Berg, J. A. & Steensma, H. Y. (1992a). Characterization of *Saccharomyces cerevisiae* mutants lacking the E1 α -subunit of the pyruvate dehydrogenase complex. *Eur J Biochem* **209**, 697–705.
- Wenzel, T. J., Migliazza, A., Steensma, H. Y. & van den Berg, J. A. (1992b). Efficient selection of phleomycin resistant *Saccharomyces cerevisiae* transformants. *Yeast* **8**, 667–668.
- Wésolowski-Louvel, M., Breunig, K. D. & Fukuhara, H. (1996). *Kluyveromyces lactis*. In *Nonconventional Yeasts in Biotechnology*, pp. 139–202. Edited by K. Wolf. Berlin & Heidelberg: Springer.
- Weusthuis, R. A., Visser, W., Pronk, J. T., Scheffers, W. A. & van Dijken, J. P. (1994). Effects of oxygen limitation on sugar metabolism in yeasts: a continuous-culture study of the Kluyver effect. *Microbiology* **140**, 703–715.
- Winkler, A. A., Bobok, A., Zonneveld, B. J. M., Steensma, H. Y. & Hooykaas, P. J. J. (1998). The lysine-rich C-terminal repeats of the centromere-binding factor 5 (Cbf5) of *Kluyveromyces lactis* are not essential for function. *Yeast* **14**, 37–48.
- Woods, D. (1984). Oligonucleotide screening of cDNA libraries. *Focus* **6**, 1–3.

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