

Prolonged selection in aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* causes a partial loss of glycolytic capacity

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Prolonged cultivation of *Saccharomyces cerevisiae* in aerobic, glucose-limited chemostat cultures (dilution rate, 0.10 h⁻¹) resulted in a progressive decrease of the residual glucose concentration (from 20 to 8 mg l⁻¹ after 200 generations). This increase in the affinity for glucose was accompanied by a fivefold decrease of fermentative capacity, and changes in cellular morphology. These phenotypic changes were retained when single-cell isolates from prolonged cultures were used to inoculate fresh chemostat cultures, indicating that genetic changes were involved. Kinetic analysis of glucose transport in an 'evolved' strain revealed a decreased K_m , while V_{max} was slightly increased relative to the parental strain. Apparently, fermentative capacity in the evolved strain was not controlled by glucose uptake. Instead, enzyme assays in cell extracts of the evolved strain revealed strongly decreased capacities of enzymes in the lower part of glycolysis. This decrease was corroborated by genome-wide transcriptome analysis using DNA microarrays. In aerobic batch cultures on 20 g glucose l⁻¹, the specific growth rate of the evolved strain was lower than that of the parental strain (0.28 and 0.37 h⁻¹, respectively). Instead of the characteristic instantaneous production of ethanol that is observed when aerobic, glucose-limited cultures of wild-type *S. cerevisiae* are exposed to excess glucose, the evolved strain exhibited a delay of ~90 min before aerobic ethanol formation set in. This study demonstrates that the effects of selection in glucose-limited chemostat cultures extend beyond glucose-transport kinetics. Although extensive physiological analysis offered insight into the underlying cellular processes, the evolutionary 'driving force' for several of the observed changes remains to be elucidated.

Received 20 August 2004

Revised 1 February 2005

Accepted 9 February 2005

INTRODUCTION

Since its invention in the 1950s, chemostat cultivation of micro-organisms (characterized by growth at a fixed rate in a well-defined and tightly controlled environment) has become a widely used cultivation mode, and has proved to be an excellent tool for quantitative physiological and functional-genomics research (Boer *et al.*, 2003; Novick &

Szilard, 1950b; Piper *et al.*, 2002). However, as already noted by Novick (Novick & Szilard, 1950a), one of the inventors of chemostat cultivation, steady-state chemostats exert a strong selective pressure and result, after prolonged cultivation, in the enrichment of evolved genotypes. Such evolutionary adaptation is undesirable when chemostats are used for the accurate physiological characterization of wild-type or mutant microbial strains. It has been proposed that, as a rule of thumb, chemostat cultivations younger than 20 generations can be used for accurate physiological analysis (Ferea *et al.*, 1999; Kubitschek, 1970).

Prolonged chemostat cultivation under defined conditions offers an excellent approach to study evolution, and has been applied to various micro-organisms and nutrient limitations (Francis & Hansche, 1972; Jansen *et al.*, 2004; Rosenzweig *et al.*, 1994; Wick *et al.*, 2002). Under nutrient-limited conditions, selection is primarily for an improved

Abbreviations: ADH, alcohol dehydrogenase; ENO, enolase; FBA, fructose-1,6-diphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HXK, hexokinase; PDC, pyruvate decarboxylase; PFK, phosphofructokinase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PYK, pyruvate kinase; RQ, respiration quotient; TPI, triosephosphate isomerase.

Supplementary expression profiles and transcriptome analysis data are available with the online version of this paper.

affinity for the growth-limiting nutrient. Any adaptation that results in a higher specific growth rate (μ) at the ambient (often vanishingly low) residual substrate concentration will result in an improved competitiveness in comparison with non-adapted cells. A general trend observed during prolonged chemostat cultivation is a progressive, generally hyperbolic, decrease of the residual concentration of the growth-limiting nutrient (Kovarova-Kovar & Egli, 1998; Senn *et al.*, 1994). This decrease reflects a selection for cells with a higher affinity [$\mu_{\max} \cdot K_s^{-1}$, in which K_s is the substrate-saturation constant for the growth-limiting nutrient (Monod, 1942), and μ_{\max} is the maximum specific growth rate]. In addition, studies on mutative adaptation of micro-organisms in chemostat cultures have demonstrated changes in cellular activity (Novick & Szilard, 1950a; van Schie *et al.*, 1989; Weikert *et al.*, 1997) and morphology (Adams *et al.*, 1985; Brown & Hough, 1965). The main challenge now resides in the identification of the molecular basis for the adaptation. Some pioneering studies with *Saccharomyces cerevisiae* exploited the availability of the complete yeast genome and of genomics tools such as DNA microarrays. Genome-wide transcriptome analysis, performed during prolonged chemostat cultivation of *S. cerevisiae* on glucose (Ferea *et al.*, 1999), revealed changes in expression of many genes, including several genes encoding proteins involved in central carbon metabolism. Furthermore, the strong selection pressure in these cultures resulted in the enrichment of mutants with one or more duplications of particular *HXT* genes, encoding high-affinity hexose transporters (Brown *et al.*, 1998). Although these studies yielded important insights into the dynamics of the yeast genome under selective conditions, the changes that were observed at the transcriptome level were not correlated to enzyme levels or metabolic capacities.

A crucial feature of bakers' yeast is its capacity to produce CO_2 , referred to as fermentative capacity (van Hoek *et al.*, 1998). After prolonged glucose-limited cultivation of *S. cerevisiae*, in addition to an increased affinity for glucose, we observed a dramatic decrease in fermentative capacity. Consequently, the aim of the present study was to perform an integral analysis of the long-term adaptation of *S. cerevisiae* during prolonged glucose-limited, aerobic cultivation in chemostat cultures, with special emphasis on the regulation of glucose transport and glycolytic capacity. To this end, we applied an integrated approach that combined transcriptome analysis, measurement of fermentative capacity and activities of glucose transport and glycolytic enzymes, and characterization of cellular morphology.

METHODS

Strains and maintenance. The haploid, prototrophic *S. cerevisiae* strain CEN.PK113-7D (*MATa MAL2-8^c SUC2*) was obtained from Dr P. Kötter, Frankfurt, Germany. Precultures were grown to stationary phase in shake-flask cultures of synthetic medium (Verduyn *et al.*, 1992), adjusted to pH 6.0 and containing 2% (w/v) glucose.

After adding sterile glycerol (30%, v/v), 2 ml aliquots were stored in sterile vials at -80°C . These frozen stock cultures were used to inoculate precultures for chemostat cultivation.

Media. Synthetic medium containing mineral salts and vitamins was prepared and sterilized as described by Verduyn *et al.* (1992). For chemostat cultivation, the glucose concentration in reservoir media was 7.5 g l^{-1} ($0.25 \text{ mol C l}^{-1}$). This medium composition has previously been demonstrated to sustain glucose-limited cultivation of *S. cerevisiae* CEN.PK113-7D (Lange & Heijnen, 2001; Verduyn *et al.*, 1992). For batch cultivation, the initial glucose concentration was 20 g l^{-1} .

Chemostat cultivation. Aerobic chemostat cultivation was performed at a dilution rate of 0.10 h^{-1} at 30°C in 1.5 l laboratory fermenters (Applikon) at a stirrer speed of 800 r.p.m. The working volume of the cultures was kept at 1.0 l by a peristaltic effluent pump coupled to an electrical level sensor. This set-up ensured that under all growth conditions, biomass concentrations in samples taken directly from the culture differed by $<1\%$ from those in samples taken from the effluent line (Noorman *et al.*, 1991). The exact working volume was measured after each experiment. The pH was kept at 5.0 ± 0.1 by an ADI 1030 biocontroller (Applikon), via the automatic addition of 2 mol KOH l^{-1} . The fermenter was flushed with air at a flow rate of 0.5 l min^{-1} using a Brooks 5876 mass-flow controller. The dissolved-oxygen concentration was continuously monitored with an oxygen electrode (model 34 100 3002; Ingold), and it remained above 60% of air saturation. Chemostat cultures were routinely checked for potential bacterial and fungal infection by phase-contrast microscopy.

Batch cultivation in fermenters. For batch cultivation in fermenters, the same set-up as for chemostat cultivation was used, except that no medium feed rate, and consequently no effluent removal rate, was applied. The starting volume of these fermentations was 1.0 l. Samples were withdrawn at appropriate intervals for determination of dry weight and metabolite concentrations.

Off-gas analysis. The exhaust gas was cooled in a condenser (2°C), and dried with a Perma Pure dryer type PD-625-12P. O_2 and CO_2 concentrations were determined with a Rosemount NGA2000 analyser. Determination of the exhaust gas flow rate and calculation of specific rates of CO_2 production and O_2 consumption were performed as described previously (van Urk *et al.*, 1988; Weusthuis *et al.*, 1994).

Determination of culture dry weight. Culture samples (10 ml) were filtered through preweighed nitrocellulose filters (pore size, $0.45 \mu\text{m}$; Gelman Sciences). After removal of medium, the filters were washed with demineralized water, dried in a Whirlpool Easytronic M591 microwave oven for 20 min at 360 W output, and weighed. Duplicate determinations varied by $<1\%$.

Extracellular metabolite analysis. Glucose, ethanol, glycerol, acetate and pyruvate present in the supernatant of chemostat cultures were determined by HPLC analysis using an HPX-87H Aminex ion-exchange column ($300 \times 7.8 \text{ mm}$, Bio-Rad) at 60°C . The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml min^{-1} . Pyruvate and acetate were detected by a Waters 441 UV-meter at 214 nm, coupled to a Waters 741 data module. Glucose, ethanol and glycerol were detected by an ERMA type ERC-7515A refractive-index detector coupled to a Hewlett Packard type 3390A integrator. Glucose concentrations in reservoir media were also analysed by HPLC.

Residual glucose measurements of continuous cultures. Samples of cells (5 ml) were rapidly (within 3 s) transferred from the chemostat culture into a syringe containing 62.0 g cold steel beads (diameter, 4 mm; temperature, -20°C) (Mashego *et al.*,

2003). After withdrawal from the fermenter, the sample was directly filtered (0.2 µm pore-size filter; Schleicher & Schuell). The supernatant was analysed for glucose by a commercial kit (catalogue no. 716251; Diffchamb Biocontrol), as described previously (Jansen *et al.*, 2002).

Fermentative capacity assays. Samples containing exactly 200 mg dry weight of biomass were harvested from a steady-state chemostat culture by centrifugation (5000 g, 3 min), and resuspended in 10 ml fivefold-concentrated synthetic medium (pH 5.6). Subsequently, these cell suspensions were introduced into a thermostat-controlled (30 °C) vessel. The volume was adjusted to 40 ml with demineralized water. After 10 min incubation, 10 ml glucose solution (100 g l⁻¹) was added, and samples (1 ml) were taken at appropriate time intervals for 30 min. The 10 ml headspace was continuously flushed with water-saturated CO₂ at a flow rate of approximately 30 ml min⁻¹. The ethanol concentration in the supernatant was analysed using a colorimetric assay (Verduyn *et al.*, 1984). Fermentative capacity can be calculated from the linear increase in ethanol concentration and is expressed as mmol ethanol produced (g dry yeast biomass)⁻¹ h⁻¹ (van Hoek *et al.*, 1998). Growth during these assays can be neglected, as no significant change in biomass concentration was observed.

Preparation of cell extracts. For preparation of cell extracts, culture samples were harvested by centrifugation, washed twice with phosphate buffer pH 7.5 (10 mM potassium phosphate, 2 mM EDTA), concentrated fourfold, and stored at -20 °C. Before assaying, the cells were thawed, washed and resuspended in phosphate buffer pH 7.5 (100 mM potassium phosphate, 2 mM MgCl₂, 1 mM DTT). Intracellular proteins were released by sonication at 0 °C using glass beads (0.7 mm diameter) in an MSE Soniprep 150 sonicator (150 W output, 8 µm peak-to-peak amplitude) for 3 min at 0.5 min intervals. Unbroken 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. In all cell extracts, this method released 53 ± 4% of the total cellular proteins.

Enzyme assays. Enzyme assays were performed with a Hitachi model 100-60 spectrophotometer at 30 °C and 340 nm (ϵ_{340} of reduced pyridine-dinucleotide cofactors 6.3 mM⁻¹) with freshly prepared cell extracts. All enzyme activities are expressed as µmol substrate converted per min per mg protein [U (mg protein)⁻¹]. When necessary, extracts were diluted in sonication buffer. All assays were performed with two concentrations of cell extract. Specific activities of these duplicate experiments differed by <10%.

Hexokinase (HXK; EC 2.7.1.1) was assayed according to Postma *et al.* (1989). Phosphoglucose isomerase (PGI; EC 5.3.1.9) was assayed according to Bergmeyer (1974), with minor modifications. The assay mixture contained: Tris/HCl buffer (pH 8.0) 50 mM, MgCl₂ 5 mM, NADP⁺ 0.4 mM, glucose-6-phosphate dehydrogenase (Roche) 1.8 U ml⁻¹ and cell extract. The reaction was started with 2 mM fructose 6-phosphate. Phosphofructokinase (PFK; EC 2.7.1.11) was assayed according to de Jong-Gubbels *et al.* (1995), with minor modifications. The assay mixture contained: imidazole/HCl (pH 7.0) 50 mM, MgCl₂ 5 mM, NADH 0.15 mM, fructose 2,6-diphosphate 0.10 mM, fructose-1,6-diphosphate aldolase (FBA; Roche) 0.45 U ml⁻¹, glycerol-3-phosphate dehydrogenase (Roche) 0.6 U ml⁻¹, triosephosphate isomerase (TPI) 1.8 U ml⁻¹ (Roche) and cell extract. The endogenous activity was measured after adding 0.25 mM fructose 6-phosphate. The reaction was started with 0.5 mM ATP. FBA (EC 4.1.2.13) was assayed according to van Dijken *et al.* (1978). TPI (EC 5.3.1.1) was assayed according to van Hoek (2000). Glycerolaldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) was assayed according to van Hoek (2000), with minor modifications. The assay mixture contained the following: triethanolamine/HCl buffer (pH 7.6) 100 mM, ATP 1 mM, EDTA 1 mM, MgSO₄ 1.5 mM, NADH 0.15 mM, phosphoglycerate kinase (PGK) 25.0 U ml⁻¹

(Sigma) and cell extract. The reaction was started with 5 mM 3-phosphoglycerate (triethylammonium salt). PGK (EC 2.7.2.3) was assayed using the same method as for GAPDH, except that PGK was replaced by glyceraldehyde-3-phosphate dehydrogenase, 8.0 U ml⁻¹ (Roche). Phosphoglycerate mutase (PGM; EC 5.4.2.1) was assayed according to Bergmeyer (1974). Enolase (ENO; EC 4.2.1.11) was assayed according to van Hoek (2000). Pyruvate kinase (PYK; EC 2.7.1.40) was assayed according to de Jong-Gubbels *et al.* (1995), with minor modifications. The assay mixture contained the following: cacodylic acid/KOH (pH 6.2) 100 mM, KCl 100 mM, ADP 10 mM, fructose 1,6-diphosphate 1 mM, MgCl₂ 25 mM, NADH 0.15 mM, lactate dehydrogenase (Roche) 11.25 U ml⁻¹ and cell extract. The reaction was started with 2 mM phosphoenolpyruvate. Pyruvate decarboxylase (PDC; EC 4.1.1.1) and alcohol dehydrogenase (ADH; EC 1.1.1.1) were assayed according to Postma *et al.* (1989).

Total RNA isolation. Cells were rapidly (within 3 s) transferred from the chemostat culture into liquid nitrogen to immediately quench the metabolism. The frozen cell suspension (about 40 g cell broth) was thawed gently on ice. After complete thawing, the cell suspension was centrifuged at 0 °C, 5000 g, for 5 min. Total RNA extraction from the pellets was performed using the hot-phenol method (Schmitt *et al.*, 1990).

Microarray analysis. The results for each growth condition were derived from three independently cultured replicates. Sampling of cells from chemostats, probe preparation, and hybridization to Affymetrix GeneChip microarrays, as well as data acquisition and analysis, were performed as previously described (Daran-Lapujade *et al.*, 2004; Piper *et al.*, 2002). Statistical analysis was performed using Microsoft Excel running the significance analysis of microarrays add-in (SAM; version 1.12; Tusher *et al.*, 2001). Genes were considered as being changed in expression if they were identified as being significantly changed by at least twofold using SAM (expected median false-discovery rate of 1%). Promoter analysis was performed using web-based software Regulatory Sequence Analysis Tools (<http://rsat.ulb.ac.be/rsat/>; van Helden *et al.*, 2000), as previously described by Daran-Lapujade *et al.* (2004). The complete dataset can be found at <http://www.bt.tudelft.nl/glucose-selection>, and the genes with significant change in expression are listed in Supplementary Figure S1 with the online version of this paper.

Image analysis. Microscopic images were taken using an Olympus IMT-2 reverse microscope, and analysed using an Olympus camera adaptor, a CCD camera, Olympus MTV-3, and the image analyser software Leica Qwin, version pro 2.2.

Restart of chemostat cultivation. A stored glycerol stock (-80 °C) of a prolonged glucose-limited chemostat cultivation culture (containing 30%, v/v, sterile glycerol) was streaked out once for purity on a synthetic medium plate containing 0.8% (w/v) glucose. Single colonies were used for inoculation of a shake-flask containing 0.8% (w/v) glucose, which, after growth to stationary phase, was used as the inoculum for the chemostat culture.

Glucose-pulse experiments. After a steady-state had been established at a dilution rate of 0.10 h⁻¹, the medium-supply and effluent-removal pumps were switched off. Immediately afterwards, 18 ml sterile 50% (w/v) glucose solution was aseptically added to the culture. At appropriate time intervals, samples were taken for measurement of metabolite concentrations, OD₆₆₀ and dry weight. Sugar concentrations and metabolite levels in the supernatants were determined by HPLC analysis.

Intracellular metabolite analysis. Samples (525 µl) for intracellular metabolite analysis were taken rapidly (within 3 s) from the fermenter, and collected on ice in perchloric acid (5%, v/v, final concentration). Samples were neutralized after 15 min by addition of 150 µl 2 M K₂CO₃ (0.35 M final concentration), and stored at

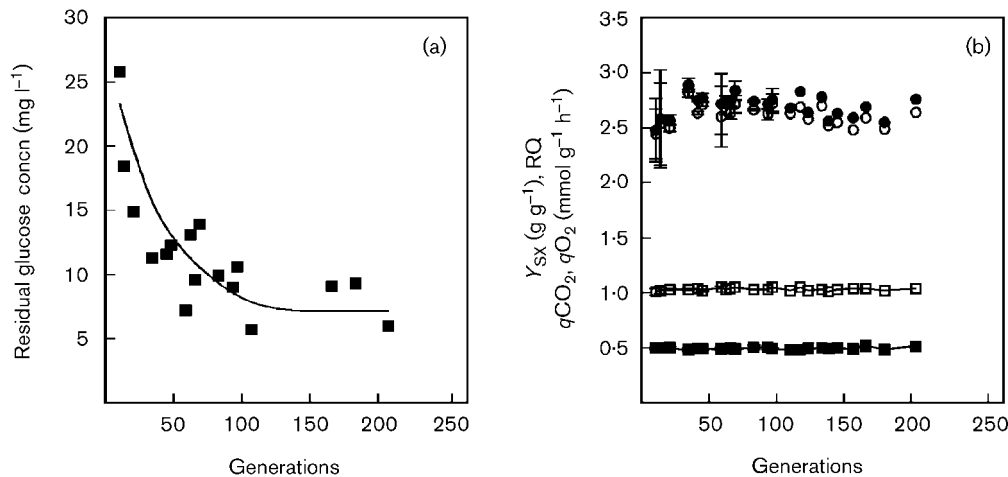


Fig. 1. (a) Residual glucose concentrations and (b) biomass yield (Y_{SX} , ■) and respiration rates (RQ, □; qCO_2 , ●; qO_2 , ○) in an aerobic, glucose-limited chemostat culture of *S. cerevisiae* CEN.PK113-7D as a function of the number of generations. Panel (a) shows the mean of two experiments with samples from independent chemostat cultures; these duplicates differed by less than 20%. Panel (b) shows means of two independent chemostat cultures; bars indicate the variation about the mean.

–20 °C. Before analysis, samples were centrifuged for 1 min at 16 000 g. Intracellular metabolites were measured on a COBAS-FARA automatic analyser (Roche). Intracellular concentrations were calculated assuming that 1 mg protein corresponds to 3.75 μ l intracellular volume (Richard *et al.*, 1996; Teusink *et al.*, 1998a). Furthermore, it was assumed that cells of the reference and the 'evolved' strains had the same cell volume.

Hexose transport assays. Cells from chemostat cultures were harvested by centrifugation at 4 °C (5 min, 5000 g), washed once in 0.1 M potassium phosphate buffer (pH 6.5), and resuspended in the same buffer to a concentration of approximately 4 g protein l⁻¹. Cells were kept on ice until further use. Zero-*trans* influx of glucose was determined at 30 °C, according to Walsh *et al.* (1994). All data fitted well to one-component kinetics.

Protein determinations. Protein concentrations in cell extracts used for enzyme analysis, and in cell suspensions used for hexose transport studies, were estimated by the Lowry method. Dried bovine serum albumin (fatty-acid free, obtained from Sigma) was used as a standard.

RESULTS

Prolonged glucose-limited cultivation leads to an increased substrate affinity

During prolonged cultivation of *S. cerevisiae* in independent, duplicate glucose-limited chemostat cultures, the residual glucose concentrations gradually decreased from around 22.8 ± 4.4 mg l⁻¹ (10–15 generations) to 7.5 ± 2.0 mg l⁻¹ after 200 generations of chemostat cultivation (Fig. 1a). This decreased residual glucose concentration is the consequence of an increased affinity ($\mu_{max} K_s^{-1}$) for the growth-limiting nutrient. No detectable changes were observed in biomass yield (Y_{SX}), specific rates of carbon dioxide production and oxygen consumption (qCO_2 and qO_2), or respiration quotient ($RQ = qCO_2/qO_2$) (Fig. 1b).

Decreased fermentative capacity due to prolonged glucose-limited chemostat cultivation

Although aerobic, glucose-limited cultures of wild-type *S. cerevisiae* strains grown at a dilution rate of 0.10 h⁻¹ do not produce ethanol *in situ*, they have a substantial fermentative capacity. This becomes apparent when cells are exposed to a high sugar concentration under anaerobic conditions (Fig. 2) (van Hoek *et al.*, 1998). Off-line measurements showed that prolonged glucose-limited cultivation led to a gradual, but strong decrease of the fermentative capacity, from 9.5 mmol (g biomass)⁻¹ h⁻¹ after 10 generations to 2 mmol (g biomass)⁻¹ h⁻¹ after 200 generations (Fig. 2).

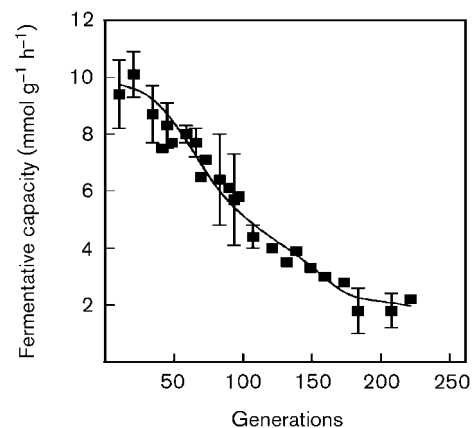


Fig. 2. Fermentative capacity of cells sampled from an aerobic, glucose-limited chemostat culture of *S. cerevisiae* CEN.PK113-7D as a function of the number of generations. Data are represented as means of two independent chemostat cultures; bars indicate the variation about the mean.

No further decrease in fermentative capacity was observed after 300 generations of chemostat cultivation (data not shown).

Correlation of enzyme levels with fermentative capacity

To analyse whether the observed decrease of fermentative capacity might be caused by changes in the levels of glycolytic enzymes, activities of individual glycolytic enzymes were assayed in cell extracts. Activities of most glycolytic enzymes showed a correlation with fermentative capacity, and decreased by two- to threefold during long-term cultivation (Fig. 3, Table 1). Exceptions were key enzymes in the upper part of glycolysis (HXK and PFK) and ADH, which showed a rather constant activity level (Fig. 3a, c). The most extreme change of the *in vitro* assayed activity was observed for ENO, the level of which decreased by almost eightfold (Fig. 3, Table 1).

Morphology

In addition to metabolic changes, changes in morphology were observed. Prolonged chemostat cultivation under glucose limitation led to a selection for more elongated cells with a reduced diameter (Fig. 4). Assuming that this pseudohyphae-like morphology did not affect the cellular volume, it resulted in an increase of the surface-to-volume ratio of the cells, thus potentially providing more space for membrane transporter proteins (Adams *et al.*, 1985). As the observed morphology was unusual for glucose-limited

cultures of *S. cerevisiae*, tests were performed to check for purity of the culture. Unpublished studies by Daran-Lapujade & Pronk have indicated that *S. cerevisiae* CEN.PK113-7D is much more sensitive to lithium ions than other yeast strains. Streaking out a sample of a 200-generation glucose-limited chemostat culture resulted in yeast colonies on non-selective plates, but not on plates containing 20 mM LiCl or more. The lithium-tolerant *S. cerevisiae* strain S288C and a stock culture of CEN.PK113-7D (non-selected sample) were included in these plate assays as positive and negative controls, respectively. This control experiment confirmed that the observed morphological and physiological changes were not due to a contamination. Furthermore, microarray studies confirmed the absence of detectable transcript levels for a number of *S. cerevisiae* genes that were previously shown to be absent in the CEN.PK113-7D background (Daran-Lapujade *et al.*, 2004).

Long-term adaptation involves stable mutations

To test if the observed changes were caused by mutations, a new chemostat was started with a single colony isolated from the prolonged chemostat cultivation under glucose limitation after 200 generations. After 10 generations of chemostat cultivation under glucose limitation of this single-cell isolate, samples were taken and analysed for fermentative capacity, residual glucose concentration and *in vitro* enzyme activities. The fermentative capacity was the same as after 200 generations of chemostat cultivation

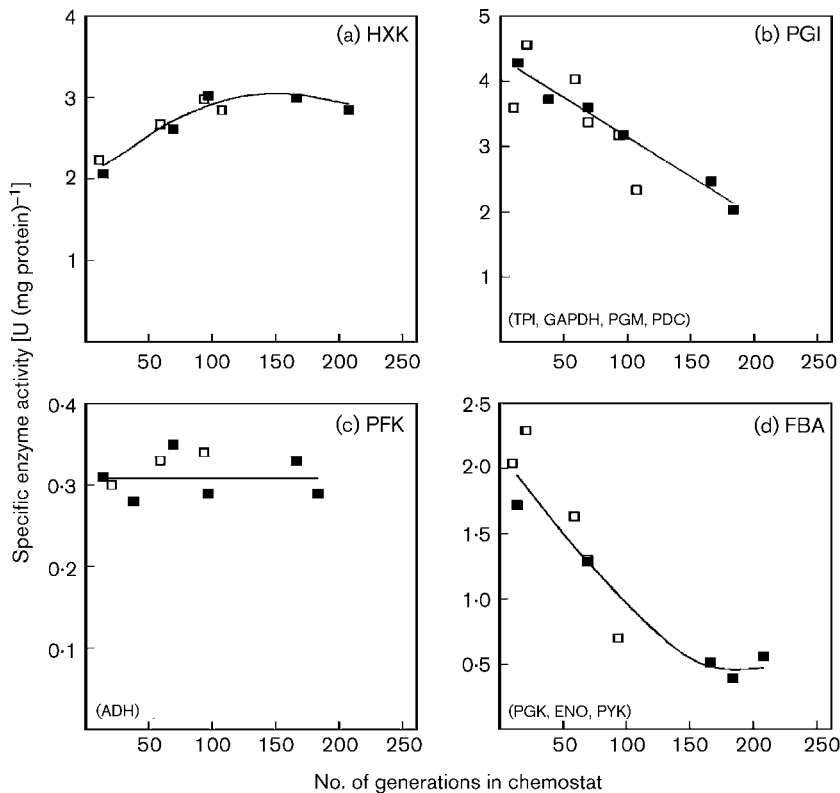


Fig. 3. Enzyme activities of cells harvested from aerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D as a function of the number of generations. Data of two experiments with cells from independent chemostat cultures (open and filled symbols) are represented. These duplicates differed by <10%. Typical profiles are represented; enzymes displaying a similar profile are shown in parentheses in the respective panel.

Table 1. Comparison of *in vitro* enzyme activities and transcript levels of cells harvested from aerobic, glucose-limited chemostat cultures of the parental strain *S. cerevisiae* CEN.PK113-7D and an evolved strain obtained after 200 generations of selection in aerobic, glucose-limited chemostat cultures

Enzyme activities are represented as means \pm the variation from the mean of two independent chemostat cultures (parental strain) or as means \pm SD of three independent chemostat cultures (evolved strain). Transcript levels are represented as means \pm SD of three independent chemostat cultures. Fold changes were calculated and evaluated for significance using Student's *t*-test (*P* values of ≤ 0.05 are considered significant, and shown in bold).

Enzyme	Specific activity [U (mg protein) ⁻¹]		Fold change	<i>P</i>	Gene name	Transcript level		Fold change	<i>P</i>
	Parental strain	Evolved strain				Parental strain	Evolved strain		
HXK	2.15 \pm 0.12	2.84 \pm 0.72	1.3	0.23	<i>HXK1</i>	1562.6 \pm 158.9	1350.0 \pm 484.6	-1.2	0.53
					<i>HXK2</i>	885.3 \pm 112.8	833.1 \pm 175.6	-1.1	0.69
					<i>GLK1</i>	1512.3 \pm 314.8	1729.5 \pm 325.5	1.1	0.45
PGI	3.93 \pm 0.48	2.14 \pm 0.20	-1.8	0.09	<i>PGI1</i>	1851.7 \pm 129.1	1066.5 \pm 37.3	-1.7	0.01
PFK	0.31 \pm 0.01	0.27 \pm 0.03	-1.1	0.18	<i>PFK1</i>	751.8 \pm 93.1	418.7 \pm 106.3	-1.8	0.02
					<i>PFK2</i>	898.5 \pm 38.4	573.5 \pm 52.8	-1.6	0.00
FBA	1.88 \pm 0.23	0.61 \pm 0.24	-3.1	0.02	<i>FBA1</i>	3584.7 \pm 98.0	2589.7 \pm 511.2	-1.4	0.07
TPI	82.22 \pm 8.70	41.65 \pm 6.52	-2.0	0.04	<i>TPI1</i>	3689.7 \pm 249.1	3040.0 \pm 434.9	-1.2	0.11
GAPDH	6.24 \pm 0.29	2.18 \pm 0.82	-2.9	0.01	<i>TDH1</i>	1557.3 \pm 173.5	262.6 \pm 319.9	-5.9	0.01
					<i>TDH2</i>	3623.3 \pm 321.9	2423.0 \pm 300.5	-1.5	0.01
					<i>TDH3</i>	4299.9 \pm 339.8	3232.6 \pm 485.6	-1.3	0.04
PGK	11.52 \pm 0.77	3.78 \pm 1.50	-3.0	0.00	<i>PGK1</i>	3474.4 \pm 339.6	1963.8 \pm 341.9	-1.8	0.01
PGM	10.82 \pm 1.06	3.32 \pm 2.24	-3.3	0.02	<i>GPM1</i>	3107.5 \pm 128.4	1963.5 \pm 501.8	-1.6	0.05
					<i>GPM2</i>	71.4 \pm 2.3	59.6 \pm 22.5	-1.2	0.46
					<i>GPM3</i>	36.7 \pm 4.8	53.7 \pm 9.5	1.5	0.07
ENO	1.04 \pm 0.10	0.14 \pm 0.07	-7.6	0.01	<i>ENO1</i>	3737.5 \pm 547.0	1116.2 \pm 683.9	-3.3	0.01
					<i>ENO2</i>	2509.3 \pm 299.3	982.4 \pm 99.6	-2.6	0.01
PYK	3.88 \pm 1.61	2.4 \pm 0.19	-1.6	0.42	<i>PYK1</i>	2104.4 \pm 400.4	945.2 \pm 296.5	-2.2	0.02
					<i>PYK2</i>	51.4 \pm 14.2	59.6 \pm 16.2	1.2	0.55
PDC	0.62 \pm 0.01	0.14 \pm 0.05	-4.5	0.00	<i>PDC1</i>	1462.0 \pm 138.7	672.6 \pm 116.0	-2.2	0.00
					<i>PDC2</i>	96.1 \pm 20.1	91.1 \pm 13.4	-1.1	0.74
					<i>PDC5</i>	94.9 \pm 25.4	102.8 \pm 16.9	1.1	0.68
					<i>PDC6</i>	80.4 \pm 31.1	34.8 \pm 36.1	-2.3	0.17
ADH	8.31 \pm 0.26	8.73 \pm 1.95	1.1	0.75	<i>ADH1</i>	2934.9 \pm 105.8	1653.5 \pm 178.2	-1.8	0.00
					<i>ADH2</i>	4616.3 \pm 129.5	4018.0 \pm 532.8	-1.1	0.19
					<i>ADH3</i>	625.3 \pm 127.1	828.6 \pm 143.7	1.3	0.14
					<i>ADH4</i>	116.8 \pm 39.8	160.7 \pm 23.7	1.4	0.19
					<i>ADH5</i>	197.8 \pm 30.6	175.9 \pm 68.0	-1.1	0.65

under glucose limitation [2.0 ± 0.2 mmol (g dry biomass)⁻¹ h⁻¹], and the residual glucose concentration showed the same reduced level (7.9 ± 2.1 mg l⁻¹). Also, glycolytic

enzyme activity levels were comparable with those measured after 200 generations of chemostat cultivation under glucose limitation (Table 1, Fig. 3). Since the isolation of the

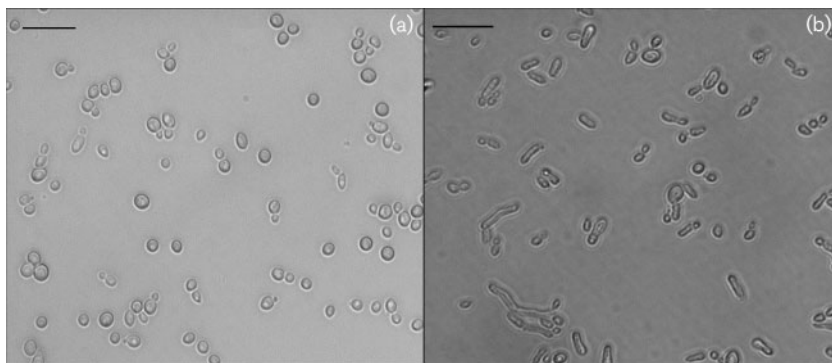


Fig. 4. Morphology of cells harvested from an aerobic, glucose-limited chemostat culture of *S. cerevisiae* CEN.PK113-7D as a function of the number of generations. (a) Sample taken after 10 generations. (b) Sample taken after 110 generations.

single-cell line and the subsequent restart of the chemostat involved cultivation under non-selective glucose-excess conditions, these results demonstrate the involvement of stable genetic changes.

Prolonged glucose-limited chemostat cultivation results in altered glucose uptake kinetics

The evolved strain exhibited a lower K_m (0.54 ± 0.07 mM) and a higher V_{max} [865 ± 39 nmol min⁻¹ (mg protein)⁻¹] for glucose uptake than the reference strain [K_m 1.54 ± 0.23 mM, and V_{max} 551 ± 4.5 nmol min⁻¹ (mg protein)⁻¹]. These kinetic data for glucose transport were consistent with the higher affinity for glucose of the evolved strain as reflected by the lower residual glucose concentration in the chemostat cultures. However, the increased capacity of glucose transport in the evolved strain indicates that glucose transport does not control its strongly reduced fermentative capacity.

Transcript levels of glycolytic genes, but not HXT genes, correlated with activity assays

To assess whether the major changes in glycolytic enzyme levels and glucose-uptake kinetics were controlled at the level of transcription, transcript levels of relevant structural genes were analysed with DNA microarrays. A Student's *t*-test was used to assess the statistical significance of the observed changes (Tables 1 and 2). For most glycolytic enzymes, the changes in enzyme levels observed in cell extracts were qualitatively consistent with changes at the mRNA level. Notable exceptions were PFK, FBA, TPI and ADH. Transcript levels of PFK and ADH were lower in the evolved strain, whereas *in vitro* enzyme activities remained constant. On the other hand, FBA and TPI showed a downregulation of *in vitro* enzyme activities, but no significant change at the mRNA level. Quantitatively, the correlation between changes in transcript level and *in vitro* enzyme activity did not always match. Changes in transcript levels for GAPDH, PGK, PGM, ENO and PDC were less strong than the changes in *in vitro* enzyme activity. These observations suggest that the levels of some glycolytic enzymes are partially controlled at a post-transcriptional level.

S. cerevisiae harbours over 20 genes with sequence similarity to hexose transporters. The observed decrease of the K_m for glucose transport could not be correlated with an increased expression of any of the known 'high-affinity' glucose transporters (Hxt2p, Hxt6p or Hxt7p; Table 2) (Bisson, 1988; Ramos *et al.*, 1988). Of the major glucose transporters (Hxt1p–Hxt7p) present in *S. cerevisiae*, only *HXT5*, which encodes a transporter with intermediate affinity ($K_m \sim 10$ mM), showed a significantly decreased transcript level (Table 2) (Diderich *et al.*, 2001).

Unbiased transcriptome analysis

To investigate whether, in addition to the transcription of glycolytic genes, transcription of other genes was affected in

Table 2. Comparison of glucose-transport-related transcript levels of cells harvested from aerobic, glucose-limited chemostat cultures of the parental strain *S. cerevisiae* CEN.PK113-7D and an evolved strain obtained after 200 generations of selection in aerobic, glucose-limited chemostat cultures

Transcript levels are represented as means \pm SD of three independent chemostat cultures. Fold changes are calculated and evaluated for significance using Student's *t*-test (*P* values of ≤ 0.05 are considered significant, and shown in bold).

Gene	Transcript level		Fold change	<i>P</i>
	Parental strain	Evolved strain		
<i>HXT1</i>	15.5 \pm 1.2	13.4 \pm 1.2	-1.2	0.10
<i>HXT2</i>	1839.6 \pm 64.8	1623.4 \pm 518.0	-1.1	0.55
<i>HXT3</i>	91.4 \pm 28.6	105.7 \pm 10.2	1.2	0.48
<i>HXT4</i>	87.4 \pm 15.9	65.8 \pm 89.9	-1.3	0.72
<i>HXT5</i>	1172.3 \pm 174.2	362.7 \pm 42.7	-3.2	0.01
<i>HXT6</i>	2936.7 \pm 233.2	2582.7 \pm 318.8	-1.1	0.20
<i>HXT7</i>	2648.4 \pm 277.8	2526.1 \pm 238.2	-1.0	0.59
<i>HXT8</i>	12.9 \pm 1.6	16.5 \pm 4.2	1.3	0.27
<i>HXT9</i>	12.5 \pm 0.8	12.9 \pm 1.6	1.0	0.69
<i>HXT10</i>	28.6 \pm 7.8	20.1 \pm 7.0	-1.4	0.23
<i>HXT12</i>	22.7 \pm 15.8	20.7 \pm 4.5	-1.1	0.85
<i>HXT14</i>	12	12	1.0	–
<i>HXT16</i>	12.2 \pm 0.3	59.9 \pm 30.5	4.9	0.11
<i>RGT2</i>	43.7 \pm 8.2	32.5 \pm 7.0	-1.3	0.15
<i>SNF3</i>	25.9 \pm 1.4	34.4 \pm 7.3	1.3	0.18
<i>GAL2</i>	12	12	1.0	–
<i>STL1</i>	69.0 \pm 1.1	23.6 \pm 6.9	-2.9	0.01
<i>MTH1</i>	114.7 \pm 14.1	188.0 \pm 52.2	1.6	0.13
<i>STD1</i>	34.4 \pm 7.1	64.7 \pm 21.4	1.9	0.12
<i>YDL247W</i>	16.8 \pm 4.3	42.9 \pm 51.5	2.6	0.47
<i>YJR160C</i>	37.9 \pm 12.0	91.5 \pm 89.7	2.4	0.41

the evolved strain, a genome-wide transcript analysis was performed. Statistical analysis identified 249 transcripts (4.1 % of the genome) whose levels significantly differed in the evolved and parental strains. Among the 186 genes that yielded a higher transcript level in the evolved strain, several were involved in cell cycle and DNA processing (34 genes, 18 %). Many of these are crucial for cell cycle progression or are involved in cell morphology (see Supplementary Fig. S1 with the online version of this paper). Upregulation of these genes may contribute to the decreased maximum specific growth rate of the evolved strain in batch cultures (Table 4, see following section) and its elongated morphology. Among the 63 genes that showed a reduced transcript level in the evolved strain, the most interesting encode proteins involved in metabolism (20 genes, 32 %), including the glycolytic enzyme genes *ENO1*, *ENO2*, *TDH1*, *PYK1* and *PDC1*. Remarkably, eight additional genes were involved in stress response (*HSP30*, *YRO2*, etc.; for complete list see Supplementary Fig. S1).

It is well known that strong selective pressure may result in

Table 3. Overrepresented sequences retrieved from the promoters of co-regulated genes in the evolved strain

Regulatory cluster	Promoter element*	No. of genes	Overrepresentation factor†	Putative binding protein
Upregulated	wCGCGwC	22	2·8	MBF (Mbp1p/Swi6p) (Iyer <i>et al.</i> , 2001)
	GGAATGC	15	3·8	?
	yrCACCCA	13	3	Aft1p/Aft2p (Rutherford <i>et al.</i> , 2003)
Downregulated	TAAGGGG	11	4·2	Msn2p/Msn4p (Martinez-Pastor <i>et al.</i> , 1996)

*Redundant nucleotides are indicated as follows: r is A or G, y is C or T, and w is A or T.

†Overrepresentation of the motif in the regulatory cluster compared to its genome coverage.

amplification or deletion of parts of chromosomes (Brown *et al.*, 1998; Dunham *et al.*, 2002). To investigate the molecular basis for the observed changes in transcript levels, up- and downregulated genes were sorted by their chromosomal location. We could indeed identify three potentially duplicated regions that together encompassed 69 of the 186 genes that showed a more than twofold higher transcript level in the evolved strain (see Supplementary Table 1 with the online version of this paper). The promoter regions of the remaining 180 genes were searched for overrepresented sequences in order to see if co-regulation was the result of binding by specific transcription factors. The analysis of upregulated genes recovered three short overrepresented sequences (Table 3). The most relevant sequence was wCGCGwC, which matched the well-described binding sites for the MBF complex (ACGCGn; Iyer *et al.*, 2001). This complex is involved in cell cycle progression control, and many of its targets were indeed upregulated in the evolved strain. From the downregulated genes, the only overrepresented sequence (TAAGGGG) (Table 3) contains the core of the stress-response element (AGGGG; Martinez-Pastor *et al.*, 1996), in good agreement with the downregulation of several stress-related genes. Despite the clear downregulation of several glycolytic transcripts, the binding site for Gcr1p, a key glycolytic transcription factor, was not significantly overrepresented (1·2-fold). This may be due to the very high genome coverage (72%) of its CwTCC core sequence (Chambers *et al.*, 1995). It is noteworthy that *TYE7*, encoding a transcription factor previously associated with activation of glycolytic genes (mainly *ENO1* and *ENO2*; Nishi *et al.*, 1995), was 2·6-fold lower in the evolved strain.

The evolved strain exhibits a delayed aerobic fermentation response

Even under fully aerobic conditions, wild-type *S. cerevisiae* strains instantaneously produce ethanol when exposed to excess glucose, a phenomenon known as the 'short-term Crabtree effect' (Rieger *et al.*, 1983; van Urk *et al.*, 1990). To check how the evolved strain reacts to glucose excess under aerobic conditions, a 50 mM glucose pulse was added directly to steady-state glucose-limited chemostat cultures. In contrast to the parental strain CEN.PK113-7D, the

evolved strain did not exhibit instantaneous ethanol production after the glucose pulse, although glucose uptake did occur (Fig. 5). Consistent with the occurrence of an exclusively respiratory glucose metabolism, the RQ remained close to unity during this period. Although ethanol production did occur after a delay of ~90 min, the specific rate of glucose consumption remained lower than in the cultures of the reference strain. Production of other, minor metabolites (acetate, glycerol) showed a similar delay (data not shown).

Glycolytic enzyme activities (Table 1) suggested that the reduced glycolytic capacity of the evolved strain might be primarily due to a reduced level of enzymes in the lower half of glycolysis. An overcapacity of the initial reactions in

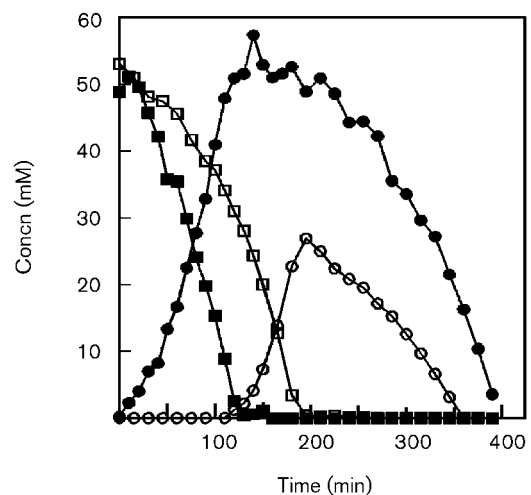


Fig. 5. Response of glucose-limited, aerobic chemostat cultures (dilution rate, 0·1 h⁻¹) to a 50 mM glucose pulse. Filled symbols represent data of a 'young' culture of the parental strain CEN.PK113-7D (10 generations of chemostat cultivation). Open symbols represent data of a restarted chemostat cultivation of an evolved strain of CEN.PK113-7D (obtained after 200 generations selection in aerobic, glucose-limited chemostat cultures). Duplicate experiments with independent chemostat cultivations differed by <10%. □, ■, Glucose; ○, ●, ethanol. Data for the parental strain were taken from Flikweert *et al.* (1999).

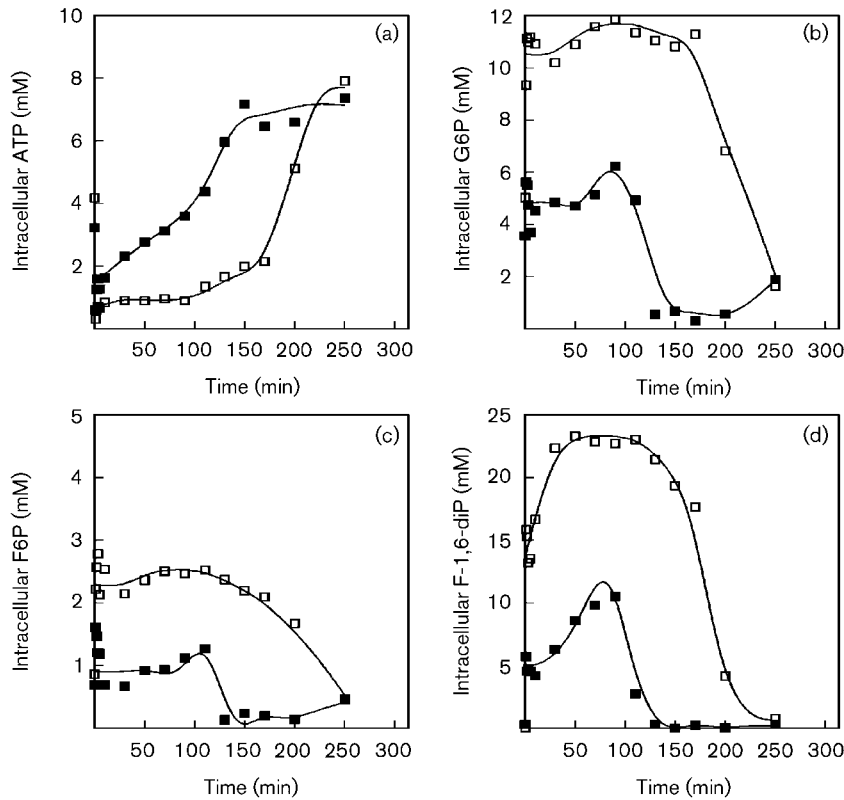


Fig. 6. Response of glucose-limited, aerobic chemostat cultures (dilution rate, 0.1 h^{-1}) to a 50 mM glucose pulse. Intracellular metabolite concentrations. Filled symbols represent data of a 'young' culture of the parental strain CEN.PK113-7D (10 generations of chemostat cultivation). Open symbols represent data of a restarted chemostat cultivation of an evolved strain of CEN.PK113-7D (obtained after 200 generations selection in aerobic, glucose-limited chemostat cultures). Duplicate experiments with independent chemostat cultivations differed by $< 10\%$. (a) ATP, (b) glucose 6-phosphate, (c) fructose 6-phosphate, (d) fructose 1,6-diphosphate.

glycolysis relative to the reactions in the lower half of glycolysis may result in depletion of ATP and build-up of hexose phosphates (Mäenpää *et al.*, 1968; Teusink *et al.*, 1998b). To check whether this mechanism might contribute to the decreased fermentation rates of the evolved strain, intracellular metabolite concentrations were monitored after a glucose pulse (Fig. 6). Consistent with the proposed mechanism, the intracellular ATP concentration after a glucose pulse was significantly lower in the evolved strain than in the parental strain CEN.PK113-7D (Fig. 6a), and the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate were significantly higher (Fig. 6b–d). Especially fructose 1,6-diphosphate accumulated to a very high concentration in the evolved strain (Fig. 6d). After consumption of the added glucose ($t=170 \text{ min}$), intracellular ATP concentrations reached the same level in the two strains (Fig. 6a).

In addition to a rapid fermentative response to aerobic glucose-excess conditions, wild-type strains of *S. cerevisiae* exhibit alcoholic fermentation during balanced aerobic growth on excess glucose. To analyse this long-term response, growth of the evolved and parental strains was compared in aerobic batch cultures. Under these conditions, a small decrease was observed with respect to ethanol production in the evolved strain (Table 4). Apparently, prolonged incubation under glucose-excess conditions enabled the induction of fermentative metabolism in

the evolved strain, consistent with the delayed fermentative response in aerobic glucose-pulse experiments. These batch experiments did reveal a difference in μ_{\max} . The evolved strain showed a μ_{\max} of 0.28 h^{-1} in fermenter cultures, whereas the reference strain had a μ_{\max} of 0.37 h^{-1} under the same cultivation conditions (Table 4).

Table 4. Growth characteristics of the parental and evolved strains in aerobic glucose-grown batch cultures

Maximum specific growth rates (h^{-1}), specific rates of glucose consumption and ethanol production [q ; $\text{mmol (g dry biomass)}^{-1} \text{ h}^{-1}$] and biomass yield (Y_{sx}) of the parental strain *S. cerevisiae* CEN.PK113-7D and the evolved strain (selection for 200 generations in chemostat under glucose limitation) in batch cultivations determined from the exponential growth phase in aerated, pH-controlled fermenters.

	CEN.PK113-7D*	Evolved strain
μ_{\max}	0.37 ± 0.01	0.28 ± 0.001
q_{s}	15.90 ± 0.14	13.59 ± 0.32
q_{ethanol}	24.70 ± 0.00	19.74 ± 0.85
Y_{sx}	0.127 ± 0.001	0.114 ± 0.002

*Data for growth characteristics of CEN.PK113-7D were obtained from van Maris *et al.* (2001).

DISCUSSION

Selection in glucose-limited chemostat cultures of *S. cerevisiae*

In agreement with previous reports (Adams *et al.*, 1985; Ferea *et al.*, 1999; Kovarova-Kovar & Egli, 1998), the present study shows that long-term glucose-limited growth of *S. cerevisiae* selects for strains with improved affinity for glucose. We demonstrate that this improved affinity is primarily caused by a strongly reduced K_m of glucose transport. An in-depth physiological analysis revealed that changes in glucose metabolism were not restricted to glucose uptake, but that prolonged glucose-limited cultivation led to a reproducible, strong decrease of the fermentative capacity. Analysis of enzyme activities in cell extracts indicated that this partial loss of fermentative capacity coincided with strongly decreased capacities of enzymes in the lower half of glycolysis. Intracellular metabolite analysis after exposure of cultures to glucose excess indicated that a resulting imbalance with the reactions in the upper half of glycolysis led to reduced intracellular ATP levels.

A possible explanation for the decreased glycolytic enzyme levels in the evolved strain is that glucose-limited cultivation provides a selective pressure to economize on protein synthesis, which is an energetically expensive process (Forrest & Walker, 1971; Oura, 1972; Stouthamer, 1973). In *S. cerevisiae*, glycolytic enzymes represent around 10–15% of the total cellular protein during aerobic, sugar-limited growth (van Hoek, 2000). The estimated change in total cellular protein represented by glycolysis in the evolved strain is ~7% relative to the parental strain. Although this decrease is too small to be clearly reflected in the biomass yield on glucose (Fig. 1b), it may well be significant during long-term selection. The observation that levels of enzymes in the upper half of glycolysis, such as HXK, were not affected may reflect their involvement in maintaining a low intracellular glucose concentration and, consequently, a high affinity of *in vivo* glucose uptake (Bisson & Fraenkel, 1983; Teusink *et al.*, 1998a).

An interesting implication of the present study is that the evolved strain was capable of growing on glucose at the same specific growth rate in chemostat and with the same biomass yield on glucose as the parental strain, even though levels of key glycolytic enzymes were drastically different. The most probable explanation is that levels of important glycolytic intermediates and/or low-molecular-weight effectors of glycolytic enzymes were different in the two strains. In addition to being of fundamental interest, this might provide an attractive means of challenging kinetic models of glycolysis. This option has been investigated in a separate study (Mashego *et al.*, 2005).

Transcriptome analysis as a tool for studying selected strains

DNA-microarray analysis provides quantitative, reproducible and genome-wide data on mRNA levels. Such analyses can, in principle, be used to investigate the molecular basis for phenotypic differences between different microbial strains, and to study the selective pressures to which micro-organisms are exposed in nature, in the laboratory or in industry. However, our results underline some inherent limitations of this approach.

Although a major change in glucose-uptake kinetics was observed in the evolved strain, this could not be clearly attributed to a different transcript level of any of the known *HXT*-encoded glucose transporters, nor did the observed K_m coincide with that of any of the known hexose transporters in *S. cerevisiae* (Boles & Hollenberg, 1997; Özcan & Johnston, 1999; Reifemberger *et al.*, 1995). Interestingly, a similar high-affinity glucose-transport system has been found in *hvk2* mutants of this yeast (Petit *et al.*, 2000). Several mechanisms may be responsible for the observed reduction of the K_m , including point mutations in the structural genes, involvement of other proteins, and changes in membrane composition.

A recent study in which glycolytic fluxes and transcript levels of glycolytic genes were compared in chemostat cultures grown on different carbon sources demonstrated that glycolytic mRNA levels are poor indicators for glycolytic flux (Daran-Lapujade *et al.*, 2004). The present study shows that this conclusion also holds for a comparison between different *S. cerevisiae* strains grown under identical conditions. Indeed, the reduced glycolytic enzyme activities could not be fully correlated to a decrease in transcription, indicating that modifications in post-transcriptional processes were also involved in the selection process.

A non-biased transcriptome analysis yielded a large number of genes that showed a significantly different transcript level in the parental and evolved strains. Most of these changes in expression could not be linked to phenotype, and the transcriptome analysis clearly failed to identify the molecular basis of the evolution. In this work, we isolated three single-cell lines from a single prolonged chemostat culture, resulting in highly similar evolved strains. Ideally, statistical analysis of a large number of independent selection experiments would reveal whether selection ultimately converges to the same or similar genotypes, or whether different genotypes may become dominant. In the latter case, transcriptome analysis would probably be more helpful in identifying the mutations responsible for the evolved phenotype. Ferea *et al.* (1999) used independently selected aerobic glucose-limited cultures of *S. cerevisiae* for transcriptome analysis, and could indeed identify a somewhat smaller set of 88 transcripts with significantly changed expression between short- and long-term cultivations. They

also observed a decreased expression of genes encoding enzymes in the lower part of glycolysis, indicating that this feature represents a significant competitive advantage for *S. cerevisiae* grown under aerobic glucose limitation. However, most of the other genes with changed expression do not overlap between this study and that of Ferea *et al.* (1999). This is probably due to the use of slightly different culture set-up between the two studies (different dilution rate, different strain, different metabolism).

Implications for biotechnological application and evolutionary engineering of *S. cerevisiae*

Chemostats are perfectly suited tools for strain improvement via evolutionary engineering, thus providing an appealing alternative to empirical strain improvement, and a valuable addition to metabolic engineering approaches. The principle of evolutionary engineering is to confront a micro-organism with a certain environment, and let natural selection 'engineer' its genome until mutants with the desired phenotype (novel catabolic activity, improved stress resistance, etc.; for review see Sauer, 2001) take over the culture. Clearly, the strongly reduced fermentative capacity that is obtained after long-term selection in glucose-limited chemostat cultures disqualifies this procedure as a means of obtaining improved bakers' yeast strains. This study highlights the necessity to rationally design the chemostat for selection pressure and culture condition in order to direct evolution towards the desired phenotype.

Evolutionary engineering has been successfully applied to improve industrially relevant physiological properties (Flores *et al.*, 1996; Hall & Hauer, 1993; Sauer, 2001). However, during long-term glucose-limited cultivation, we observed that an improved affinity was accompanied by a strongly delayed response to glucose excess. In a recent study on evolution of *S. cerevisiae* in maltose-limited chemostat cultures, we observed a similar apparent 'trade-off' between affinity in nutrient-limited chemostat cultures and the ability to cope with a sudden exposure to sugar excess (Jansen *et al.*, 2004). These observations underline that selection, under steady-state nutrient-limited conditions, of spontaneous or induced mutants with desirable traits may come at the expense of their ability to cope with changes in the nutrient concentration. This is relevant when chemostat cultures are used for the directed selection of strains with industrially relevant properties (Flores *et al.*, 1996; Kuyper *et al.*, 2004; Sauer, 2001). Furthermore, evolutionary engineering will become a valuable tool for rationally designed metabolic engineering approaches only if the molecular basis of the desired phenotype can be identified and used to genetically engineer micro-organisms. The present work exemplifies the difficulty of discovering the mutated gene(s) responsible for adaptation, and underlines the current limitation and challenges of evolutionary engineering.

ACKNOWLEDGEMENTS

We thank Professor Hans van Dijken for many stimulating discussions. The PhD project of M.L.J. was financially supported by the Dutch Ministry of Economic Affairs via the EET programme. P.D.-L. and J.A.D. were sponsored by STW, DGC.5232. The research group of J.T.P. is part of the Kluyver Centre for Genomics of Industrial Fermentation, which is supported by the Netherlands Genomics Initiative.

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