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The onset of fermentative metabolism in continuous cultures depends on the catabolite repression properties of *Saccharomyces cerevisiae*

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In glucose-limited continuous cultures, a Crabtree positive yeast such as Saccharomyces cerevisiae *displays respiratory metabolism at low dilution rates (*D*) and respirofermentative metabolism at high* D. *We hypothesized that the onset of fermentative metabolism is related with the catabolite repression or glucose repression effect. To test this hypothesis, we have investigated the physiological behavior in glucose-limited continuous cultures of* S. cerevisiae *strain CEN.PK122 and isogenic mutants,* snf1 *(*cat1*) and* snf4 *(*cat3*), defective in proteins involved in the release from glucose repression and the mutant in glucose repression* mig1. *We analyzed the behavior of the wild type and mutant strains at steady state in chemostat cultures as a function of* D. *Wild-type cells displayed respiratory metabolism up to a* D *of 0.2* h^{-1}. snf1 *and* snf4 *mutants started fermenting after a* D *of 0.1 and 0.15* h^{-1}, *respectively. The latter behavior was not due to an impairment of respiration since their specific rate of oxygen consumption was similar or even higher than that shown by the wild type. The* snf1 *strain displayed much lower yields than the wild type and the other mutants in the whole range of* D *studied. We conclude that the onset of fermentative metabolism in yeast growing in chemostat cultures is related with glucose repression.* © *1998 Elsevier Science Inc.*

Keywords: Catabolite repression mutants; chemostat cultures; fermentation; Saccharomyces cerevisiae

Introduction

Saccharomyces cerevisiae is a Crabtree positive yeast. It exhibits fermentative metabolism even under aerobic culture conditions. In glucose-limited continuous cultures, *S. cerevisiae* will display respiratory metabolism at low dilution rates D.[1] If a pulse of glucose is given under those conditions, ethanol will be transiently accumulated. This accumulation illustrates the so-called "short-term Crabtree effect" (for a review, see Weusthuis *et al.*[2]). At high D, *S. cerevisiae* will breakdown glucose respirofermentatively and this is actually known in the literature as "long-term Crabtree effect". The highest D at which metabolism is only respiratory is known as "critical dilution rate" D_c and is strain dependent. Beyond D_c, respirofermentative metabolism is triggered.

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Several interpretations have been provided to explain this phenomenon:

i. A kinetic limitation of respiratory intermediates that enter the mitochondria,[3]
ii. A limited respiratory capacity of yeast cells growing on glucose because of an "overflow" at the level of pyruvate,[4,5] or
iii. The uncoupling effect on mitochondria by acetate being produced from pyruvate and reutilized by the tricarboxylic acid cycle.[6]

We postulate that the D at which the onset of fermentative metabolism occurs depends on the catabolite repression features of the strains under study. To test our hypothesis, we have studied a series of mutants of catabolite repression that carry disruptions in genes involved in the expression of glucose-repressible genes, namely *SNF1/CAT1/CCR1*, *SNF4/CAT3*, and *MIG1* and compared its physiological behavior with that of the wild-type strain.

SNF1 (allelic to *CAT1*, *CCR1*) has been described as a main serine-threonine protein kinase required for the expression of gluconeogenic and glyoxylate cycle genes as

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Table 1 Strains used in this work

Strain	Genotype
CEN.PK122	*MATa/MATα URA3/URA3 LEU2/LEU2 TRP1/TRP1 HIS3/HIS3 MAL2–8^c/MAL2-8^c SUC2/SUC2*
CEN.SC5 (*snf1*)	*MATa/MATα URA3/URA3 LEU2/LEU2 TRP1/TRP1 his3-Δ1/his3-Δ1 snf1::HIS3/snf1::HIS3 MAL2-8^c/MAL2-8^c SUC2/SUC2*
CEN.SC12 (*snf4*)	*MATa/MATα URA3/URA3 leu2-3, 112/leu2-3, 112 TRP1/TRP1 HIS3/HIS3 snf4::LEU2/snf4::LEU2 MAL2-8^c/MAL2-8^c SUC2/SUC2*
CEN.SC21 (*mig1*)	*MATa/MATα URA3/URA3 leu2-3, 112/leu2-3, 112 TRP1/TRP1 HIS3/HIS3 mig1::LEU2/mig1::LEU2 MAL2-8^c/MAL2-8^c SUC2/SUC2*
CEN.PK2	*MATa/MATα ura3-52/ura3-52 leu2-3, 112/leu2-3, 112 trp1-289/trp1-289 his3-Δ1/his3-Δ1 MAL2-8^c/MAL2-8^c SUC2/SUC2*
CEN.PK110–10C	*MATα URA3 LEU2 TRP1 his3-Δ1 MAL2-8^c SUC2*
CEN.PK113–7A	*MATa URA3 LEU2 TRP1 his3-Δ1 MAL2-8^c SUC2*
CEN.PK113–16B	*MATα URA3 leu2-3, 112 TRP1 HIS3 MAL2-8^c SUC2*
CEN.PK111–32D	*MATa URA3 leu2-3, 112 TRP1 HIS3 MAL2-8^c SUC2*
CEN.SC1–7A	*MATa URA3 LEU2 TRP1 his3-Δ1 snf1::HIS3 MAL2-8^c SUC2*
CEN.SC3–5C	*MATα URA3 LEU2 TRP1 his3-Δ1 snf1::HIS3 MAL2-8^c SUC2*
CEN.SC2–7B	*MATa URA3 leu2-3, 112 TRP1 HIS3 snf4::LEU2 MAL2-8^c SUC2*
CEN.SC4–7B	*MATα URA3 leu2-3, 112 TRP1 HIS3 snf4::LEU2 MAL2-8^c SUC2*

well as many other genes involved in the utilization of other carbon sources (*GAL1*, *SUC2*, *MAL2*).[9–11] *SNF4* (allelic to *CAT3*) is also required for expression of glucose repressible genes and appears to code for a protein necessary for *SNF1* function.[12] On the other hand, *MIG1* encodes a zinc finger protein involved in the repression by glucose of genes implicated in the utilization of sugars such as *SUC2*, *MAL*, and *GAL* genes.[12–14] The current model about the molecular mechanism of release from glucose repression postulates that Snf1 together with Snf4 in the phosphorylated state activates the expression of the glyoxylate cycle and gluconeogenic enzymes and inhibits the function/expression of Mig1. In turn, Mig1 represses the expression of other sugar-utilizing enzymes and respiratory electron carrier proteins.

By combining the tools of genetics and physiology, we try to unravel the bases of the shift between respiratory and respirofermentative types of glucose breakdown in yeast continuous cultures. A main advantage of this approach is to study the role of *SNF1*, *SNF4*, and *MIG1* on the onset of fermentative metabolism in an isogenic background under controlled environmental conditions.

Materials and methods

Culture conditions

S. cerevisiae strain CEN.PK122 and the glucose repression mutants were grown under glucose limitation in a Biostat® B (B. Braun Biotech International) 2-l fermenter with a working volume of 1 l, a stirrer speed of 600 rpm, at 30°C and an airflow of 1.5 ± 0.2 l min^{-1}. The dissolved O_2 tension was higher than 60% air saturation at all *D* and for all strains assayed. The pH was controlled at 5.0 by addition of 2 M KOH. One liter of the medium contained: 10 g glucose, 5 g $(NH_4)_2SO_4$, 0.5 g $MgHPO_4$, 1 g KH_2PO_4, 0.1 g $CaCl_2$, 0.1 g NaCl, 15 mg $FeCl_3$, 0.2 mg Na_2MoO_4, 0.04 mg $CuSO_4$, 0.4 mg $ZnSO_4$, 0.4 mg $MnSO_4$, 0.1 mg KI, 0.5 mg boric acid, 0.4 mg Ca pantothenate, 0.4 mg niacin, 2 mg inositol, 20 μg biotin, 2 μg folic acid, 0.4 mg pyridoxine hydrochloride, 0.2 mg aminobenzoic acid, 0.2 mg riboflavin, and 0.4 mg thiamin hydrochloride.

Gas analysis was performed on-line with a Columbus gas analyzer system (Columbus, OH). The condenser of the fermenter air outlet was thermostated at 4°C and the exhaust gas was subjected to gas analysis in a paramagnetic O_2 and infrared CO_2 gas analyzer sampling every 15–20 min along 4–8 h. The gas analyzer was calibrated with air and nitrogen and a mixture of 5% CO_2 and 95% synthetic air. After reaching the steady state, the values of q_{O_2} and q_{CO_2} were calculated from averaging 6–9 measurements sampled along 2–3 h. The criteria to judge when a steady state was achieved were the constancy in optical density at 600 nm and in the CO_2 and O_2 composition in the gas phase measured over one residence time. Under our experimental conditions, it took 3.5–4.5 residence times to achieve the steady state.

Catabolite (de)repression deletion strains

To construct the *snf1::HIS3* null strain, the plasmid pJS78A[12] was digested with *EcoR*1 prior to transformation of strain CEN.PK2 (*Table 1*). After tetrad analysis, a *MATa* histidine prototroph was crossed with strain CEN.PK110-10C to generate a heterozygote prototrophic diploid strain CEN.SC1. Tetrad analysis of this strain, CEN.SC1, led to a haploid prototrophic strain CEN.SC1-7A (*MATa*) which was crossed with a *MAT*α segregant (CEN.SC3-5C) obtained following a similar procedure but crossing a *MAT*α segregant of the transformation with CEN.PK113-7A. The *snf1* deleted prototrophic zygote (CEN.SC5) was isolated by micromanipulation with a MSM System (Singer Instrument Co. Ltd., Watchet, Somerset, U.K.).

A *snf4::LEU2* disruptant strain was obtained by transformation of CEN.PK2 with plasmid pJS85A digested with *Hind*III.[15] A *MATa* leucine prototroph segregant was crossed with CEN.PK113-16B (*MAT*α) to render a diploid CEN.SC2. Tetrad analysis of CEN.SC2 led to a haploid prototrophic strain, CEN.SC2-7B (*MATa*) which was crossed with a *MAT*α segregant (CEN.SC4-7B) obtained following a similar procedure but crossing a *MAT*α segregant of the transformation with CEN.PK111-32D. The *snf4* disrupted prototrophic zygote (CEN.SC12) was isolated by micromanipulation (*Table 1*). To construct the *mig1* deletion diploid strain, we followed a similar procedure as with *snf1* and *snf4* deletion/disruption strains but we used instead plasmid pJN10 carrying the *mig1*-δ1::*LEU2* gene deleted in the zinc finger motifs[13] digested with *Sac*I before transformation of CEN.PK2. Strain CEN.SC21 was isolated by micromanipulation (*Table 1*).

Analysis of the correct gene deletions was performed by diagnostic PCR. Diploid analysis of *snf1/snf1* and *snf4/snf4* strains

that are unable to sporulate was performed by PCR using the method of Huxley *et al.*[16]

Detection of the correct gene disruption by PCR

For PCR, yeast cells were taken directly from YPD plates, resuspended in 100 μl 1 × PCR buffer, and subsequently incubated for 10 min at 96°C. A 50-μl analytical PCR reaction was performed according to the instructions of the manufacturer (Life Technologies, Effenstein, Germany) using 10 μl denatured yeast cells, 1 U Taq DNA polymerase, and 30 pmol of each primer. The incubation and cycling conditions were: 94°C for 5 min (hot start) followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 3 min. For analysis, 5 μl of the PCR reaction mixture were loaded on an agarose gel.

Extracellular metabolite analysis

Samples were taken from the culture vessel and rapidly filtered to measure the metabolite concentration in the exhaust culture medium. Glucose was determined through the glucose oxidase/horseradish peroxidase-coupled assay using ABTS (2,2′-azino-di-[3 ethyl-benzthiazolin] 6′ sulfonate) as substrate.[9] Ethanol was measured using yeast alcohol dehydrogenase as described previously.[17] Glycerol and acetate were measured with test kits through the glycero kinase, pyruvate kinase, and lactate dehydrogenase; and acetylCoA synthase, citrate synthase, and malate dehydrogenase-coupled enzyme assays, respectively. Boehringer Mannheim test kits number 148,270 and 148,261 were used for glycerol and acetate, respectively. Pyruvate was measured with lactate dehydrogenase as described before.[18]

Enzyme activities in cellular extracts and metabolite determination through enzymatic methods were performed in a Spectrophotometer Uvikon 941 Plus (Kontron Instruments) equipped with a temperature control unit. Invertase was measured as described previously.[9] The PEPCK activity assay was performed according to Hansen *et al.*[19] Nucleotides were determined by capillary electrophoresis.

Biomass was monitored through the dry weight determined from a 10-ml culture sample, centrifuged, and washed and dried overnight at 80°C. The precision in dry weight determinations was 8–10% for the highest D (near the washout of the fermenter) and less than 5% for all other D.

Results

Phenotypic characterization of WT and mutant strains

The *snf1*, *snf4*, and *mig1* disruption mutants were plated on different carbon sources in complete medium to confirm whether the phenotype was the expected one (*Table 2*). Neither *snf1* nor *snf4* mutants could grow on single gluconeogenic carbon sources, i.e., substrates generating ATP through respiration, namely ethanol, acetate, or glycerol or in a mixture of ethanol and glycerol. The *snf1* strain could neither grow on maltose nor on raffinose as the carbon source; however, the *snf4* strain could slowly grow on maltose and raffinose. This is likely due to the less severe phenotype of *snf4* mutants on the CEN.PK2 background. The absence of growth by *snf1* and *snf4* mutants on gluconeogenic carbon sources resulted from the inability of these strains to release the glucose repression of gluconeogenic enzymes essential to assimilate these carbon sources.[20] Diploid *snf1* and *snf4* strains were unable to sporulate (*Table 2*).

Table 2 Growth of the strains on various carbon sources

Strain	CEN.PK122	CEN.SC5	CEN.SC12	CEN.SC21
YEPF	++	++	++	++
YEPE	++	–	–	++
YEPGly	++	–	–	++
YEPAc	++	–	–	++
YEPEG	++	–	–	++
YEPM	++	–	+	++
YEPR	++	–	+	++
Sporulation	+	–	–	+

YEP stands for yeast extract and peptone supplemented with: ethanol (E), fructose (F), glycerol (Gly), acetate (Ac), a mixture of ethanol and glycerol (EG), maltose (M), and raffinose (R)

The level of enzyme activities such as invertase and PEPCK from cells grown until mid-exponential phase on glucose minimal medium and transferred to ethanol minimal medium is another discriminatory test of the phenotype of these mutants. A 3.5-fold increase in expression of invertase activity was observed in the diploid WT strain (*Figure 1A*). *snf1* and *snf4* mutants did not show invertase expression upon transfer to ethanol medium and the level of activity remained as low as that of glucose-grown cells (*Figure 1A*). On the other hand, the invertase activity of a *mig1* mutant strain corresponded to the levels of the ethanol-grown culture even in the presence of glucose (*Figure 1A*). In fact, this is the most distinctive phenotype of the *mig1* mutant: i.e., the lack of repression in the presence of glucose of other sugar-utilizing enzymes such as invertase or maltase.

Unlike the results obtained with invertase, PEPCK activity was repressed on glucose in all strains and only WT and the *mig1* mutant were able to express it on ethanol (*Figure 1B*). The repression factor was 100 and 25 times for WT and *mig1* strains, respectively.

Physiological behavior of WT and mutant strains in aerobic glucose-limited continuous cultures

Figure 2 shows the behavior of the main physiological variables of the WT strain CEN.PK122 as a function of the dilution rate, D. The glucose consumption rate, q_{Glc}, exhibited a linear dependence either before or beyond the critical dilution rate, D_c, although with different slopes. From the slope of q_{Glc} vs. D, we estimated the yield of biomass (71 g dw mol^{-1} glucose) which was somewhat lower than the value of 90 g dw mol^{-1} glucose displayed by other *S. cerevisiae* strains.[4] The yield decreased severely to a value of 19.1 g dw mol^{-1} glucose in the region of fermentative catabolism.

From a D of 0.225 h^{-1}, ethanol accumulated in the fermenter and the oxygen consumption rate, q_{O_2}, poised to a constant value of 5.02 mmol O_2 h^{-1} g^{-1} dw. The CO_2 production followed the increase in ethanol production rate accounting for a respiratory quotient (RQ) of 2.3 at $D = 0.3$ h^{-1} (*Figure 2B*). The P:O ratio calculated as Alexander and Jeffries[21] rendered an approximately constant value of 1.4

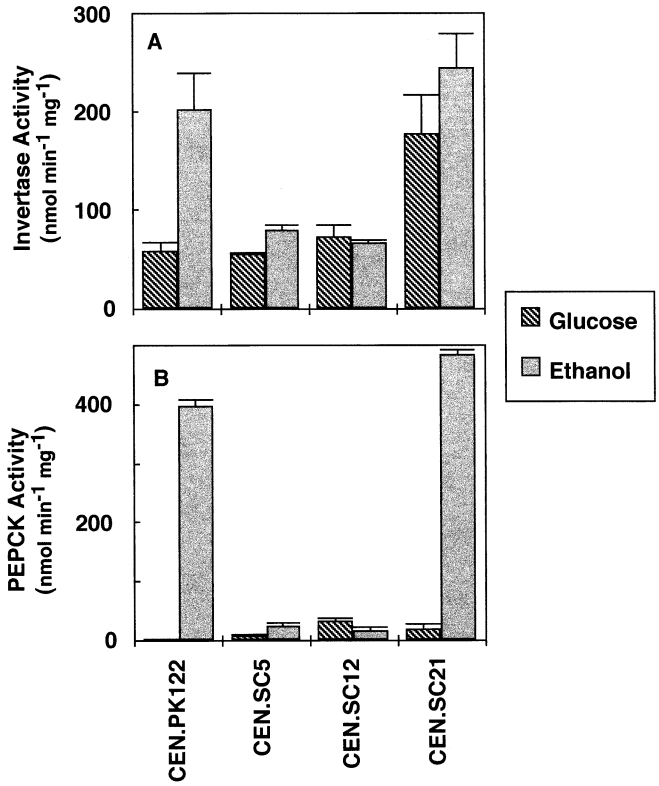

Figure 1 Invertase and PEPCK activity of the wild-type CEN.PK122 and *snf1*, *snf4*, and *mig1* mutants. Cells were grown in shake flask cultures on 4% (w/v) glucose minimal medium at 30°C and harvested at OD_{600} of 1–1.5. One half of the cultures were harvested by centrifugation and washed and transferred to 3% ethanol minimal medium where the incubation continued for 7 h at 30°C. The other half of the glucose-grown cultures as well as the ethanol-incubated ones were harvested and cells extracts prepared as described in Zimmermann *et al.*[9] Invertase and PEPCK activities in crude extracts were measured as described in MATERIALS AND METHODS and the SEM values indicated by bars were obtained from duplicates of two independent experiments

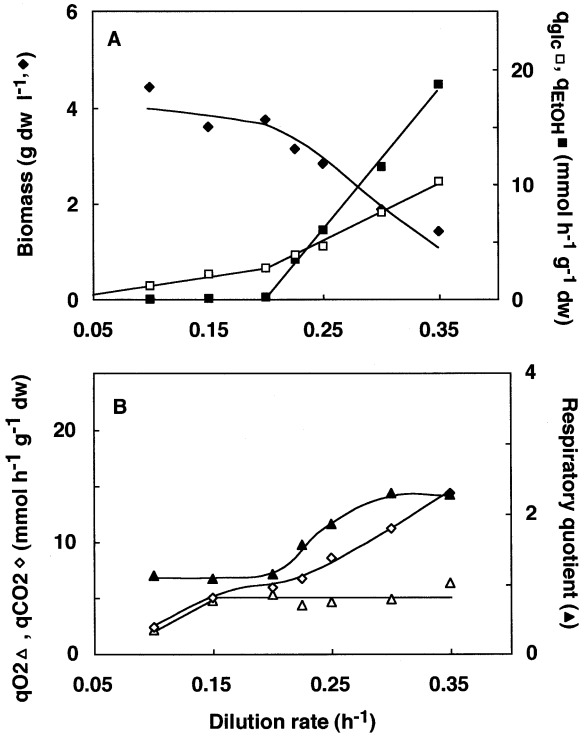

Figure 2 Physiological behavior of the wild-type strain CEN.PK122 in chemostat cultures. Biomass yield, glucose consumption, and ethanol production rates were plotted as a function of *D* after attaining the steady state judged through the constancy in biomass concentration in the reactor vessel (A). Samples of the exhaust gas were taken after the culture reached the steady state and were analyzed for their O_2 and CO_2 content as described in MATERIALS AND METHODS. The latter values were used to calculate q_{O_2}, q_{CO_2}, and *RQ* as described in MATERIALS AND METHODS. The values of all physiological variables plotted were obtained from triplicate determinations and the standard deviation was not higher than 5% of the values shown in the *Figure*

in the whole range of *D* studied. Glucose concentration in the fermenter was close to 100 μM up to a D of 0.25 h^{-1}. At higher D, it accumulated up to 12 mM at a D of 0.35 h^{-1} (not shown).

Figure 3 shows the physiological behavior of a *snf1* disrupted diploid strain. Large differences with its parental and isogenic WT are apparent. The biomass yield, Y_{Glc}, in the respiratory regime ($D \leq 0.1$ h^{-1}) was much lower (52.9 g dw mol^{-1} glucose). The respirofermentative regime started at a much lower growth rate ($D > 0.1$ h^{-1}) (*Figure 3A*). Already at 0.15 h^{-1}, the ethanol concentration in the fermenter was 10 mM; however, the *snf1* strain did not display high rates of ethanol production, q_{EtOH}, under our experimental conditions. At the highest D assayed (0.3 h^{-1}) before the washout of the fermenter, the highest q_{EtOH} recorded was 3.5 mmol h^{-1} g^{-1} dw which was fivefold lower than the 18.6 mmol h^{-1} g^{-1} dw exhibited by the WT. Consequently, the *RQ* remained at values lower than two at the highest *D* tested (*Figure 3B*). Another noticeable feature of the *snf1* strain was the behavior of respiration. In fact, q_{O_2} did not achieve a maximum at D_c but increased until a *D* of 0.2 h^{-1} amounting to 6.9 mmol h^{-1} g^{-1} dw (*Figure 3B*). Since we could not account for all the carbon consumed (*Table 3*), the P:O ratio calculations were not performed for the *snf1* strain.

Figure 4 shows the physiological behavior of the *snf4* mutant. Compared to *snf1*, the *snf4* mutant displayed a behavior closer to that of the WT strain; however, the onset of fermentative metabolism occurred after a *D* of 0.15 h^{-1} (*Figure 4A*). The Y_{Glc} during the respiratory regime was 67 g dw mol^{-1} glucose. It decreased to 39 g dw mol^{-1} glucose after the onset of the respirofermentative regime (*Figure 4A*). The behavior of q_{O_2} increased until the onset of fermentation after a *D* of 0.15 h^{-1}, displaying at a *D* of 0.25 h^{-1} a maximal q_{O_2} (7.9 mmol h^{-1} g^{-1} dw) much higher than that exhibited by the WT strain (*Figure 4B*). From the slope of variation of q_{O_2} as a function of *D* in the respiratory regime, a Y_{O_2} of 18.5 g dw mol^{-1} O_2 could be calculated. The *RQ* attained a value of 3.6 which is in agreement with the high rate of fermentation determined, 15 mmol h^{-1} g^{-1} dw (*Figure 4A*). Regarding the efficiency of the respiratory

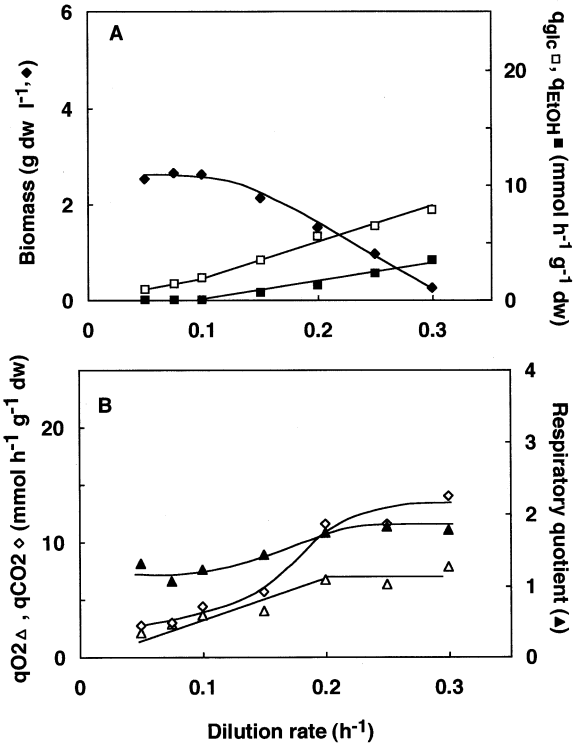

Figure 3 Physiological behavior of the *snf1* mutant in chemostat cultures. The chemostat cultures were run and analyzed as described in the legend of *Figure 2* and MATERIALS AND METHODS

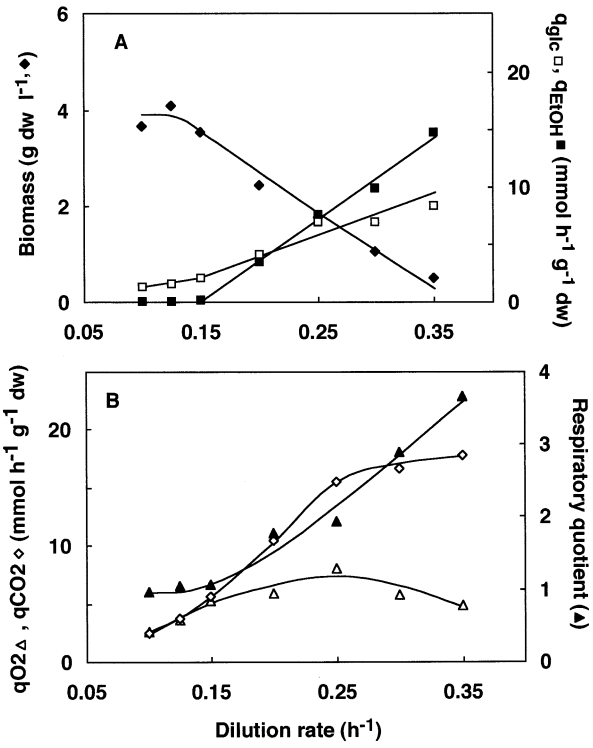

Figure 4 Physiological behavior of the *snf4* mutant in chemostat cultures. The chemostat cultures were run and analyzed as described in the legend of *Figure 2* and MATERIALS AND METHODS

chain, the *snf4* strain appears to be more repressed than the WT with an average P:O ratio of 0.78.

A *mig1* disruption mutant displayed respiratory metabolism up to a D of 0.17 h^{-1} as indicated by the RQ and the ethanol accumulation in the reactor (*Figure 5A*). A low Y_{Glc} of 63 g dw mol^{-1} glucose was observed at $D < D_c$. The *mig1* strain showed a q_{ETOH} of 20.5 mmol h^{-1} g^{-1} dw which was the highest displayed of all strains studied (*Figure 5A*). The Y_{O_2} was 26 g dw mol^{-1} O_2 which was relatively low compared to that of WT. It turns out that such a low Y_{O_2} correlated with a very low efficiency of the respiratory chain. We calculated that a P:O ratio fluctuated around 0.8 ± 0.15 in the range of D examined where Y_{ATP} values from 10–11 g dw mol^{-1} ATP had been estimated. This could explain why the *mig1* strain had such a low Y_{O_2} (comparable to the *snf4* mutant), and triggered the fermentation at such a low D, i.e., after 0.17 h^{-1}.

In the case of all mutant strains but *snf1*, the glucose concentration in the fermenter was 0.1 mM in the respiratory regime and accumulated to various extents at $D > D_c$. The level of glucose in the fermenter where the *snf1* strain was cultured at $D < D_c$ was about 0.5 mM. After a D of 0.1 h^{-1}, the steady-state glucose concentration increased until 48 mM at a D of 0.3 h^{-1} (not shown).

To estimate the carbon balance, we determined in the exhaust culture medium the steady state concentrations of ethanol, glycerol, acetate, acetaldehyde, and pyruvate at each D. The latter two metabolites accounted for less than 1% of the carbon consumed and therefore were not included in *Table 3* reporting the carbon balance at a D lower than D_c,

Table 3 Carbon balance of the wt and catabolite repression mutants growing at 30°C in aerobic glucose-limited chemostat cultures

	WT			*snf1*			*snf4*			*mig1*		
Carbon in (%)	0.15	0.2	0.3	0.05	0.1	0.25	0.125	0.15	0.25	0.1	0.15	0.225
Biomass	16.5	19.2	5.3	8.7	9.0	2.5	22.4	16.8	4.2	17.8	15.1	5.2
EtOH	1.1	1.3	53.7	0.7	0.6	12.0	0.2	2.3	36.2	0.5	0.7	47.0
CO_2	64.5	62.5	25.8	63.3	63.8	39.8	68.2	72.8	49.3	64.7	65.2	38.0
Glycerol	8.3	9.1	9.9	4.7	8.5	18.2	10.5	10.2	9.8	9.8	9.2	3.7
Acetate	3.5	2.7	1.4	3.9	1.6	2.7	0.9	2.0	1.4	0.9	3.1	1.3
C recovery	93.9	94.8	96.1	81.3	83.7	75.2	102.2	104.1	100.9	93.7	93.3	95.2

Percent of carbon recovered as various compounds at each D h^{-1} indicated in the row below the strain identification

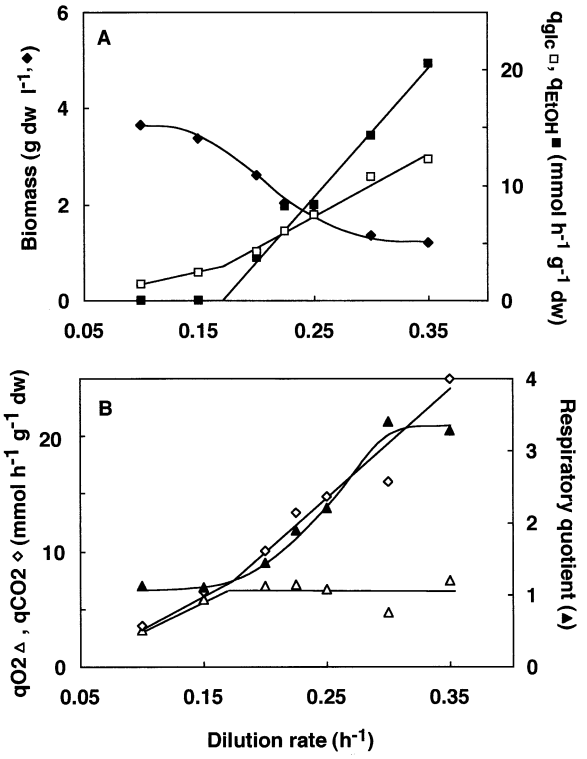

Figure 5 Physiological behavior of the *mig1* mutant in chemostat cultures. The chemostat cultures were run and analyzed as described in the legend of *Figure 2* and MATERIALS AND METHODS

at D_c and at D where the microorganisms displayed respirofermentative glucose breakdown. Under our experimental conditions, we observed a relatively constant contribution of glycerol to the carbon balance in all strains and irrespective of the type of glucose breakdown. The latter suggests that the cells likely run into a redox imbalance and produced glycerol to overcome it.

Discussion

A main contribution of the present work is to show that the onset of fermentative metabolism as a function of the growth rate ($= D$) in chemostat cultures depends upon the glucose repression features of the strain under study (*Figures 2–5*). We have compared the physiological behavior of a WT strain that under our experimental conditions displayed a D_c of 0.2 h^{-1}, with a *snf1* mutant which exhibited a D_c of 0.1 h^{-1}, a *snf4* mutant exhibiting fermentative metabolism at $D > 0.15\ h^{-1}$, *and a mig1* strain fermenting glucose at $D > 0.17\ h^{-1}$. All mutants exhibited a lower respiratory efficiency than the WT as could be judged through the P:O ratio which was calculated according to Alexander and Jeffries.[21] Comparing the q_{Glc} displayed by the various strains under study, the respirofermentative regime was triggered after q_{Glc} increased over a value of 3–4 mmol h^{-1} g^{-1} dw. This was also the case for *S. cerevisiae* H1022.[4] The effect of mutating the regulatory *SNF1*, *SNF4*, and *MIG1* genes resulted in attaining the threshold q_{Glc} at lower D (compare *Figures* 2–5, panel A).

The decrease in the estimated P:O ratio agreed with the description of the mutants performed either with punctual mutations or deletion strains as is the case in this work.[12,20] *snf1* and *snf4* mutants were not able to express succinate and NADH dehydrogenase.[20] The WT strain exhibited a P:O ratio of 1.4 that agrees well with those reported for other *S. cerevisiae* strains operating under carbon-limited chemostat cultures.[21] Isogenic mutant strain *snf4* showed a P:O ratio of 0.78. This decrease in the efficiency of oxidative phosphorylation would likely indicate a change in the number of phosphorylation sites in the respiratory chain. This point deserves further studies to confirm the protein and mRNA levels of respiratory enzymes. The modulation of the expression of the *CYC1* gene coding for isocytochrome c has been shown to be under the transcriptional control of *SNF1/SNF4* genes.[12] When Y_{ATP} was calculated, the differences between strains were lower than those exhibited by the P:O ratio, i.e., a Y_{ATP} of 10–11 for WT, 12–13 for *snf4*, and 10–12 for the *mig1* mutant.

Interestingly, we did not find changes in the P:O ratio with D which would argue in favor of a constant expression of respiratory enzymes. Besides, the measurement of gluconeogenic enzymes in extracts from cells harvested at different Ds showed a repressed pattern of activity even in the respiratory regime (not shown). In agreement with our results, other reports described that the activities and mRNA levels of glucose-repressible enzymes such as invertase did not show significant changes at increasing growth rates.[22] The latter was interpreted to indicate that the Crabtree effect was not related with glucose repression. On the contrary, the results presented here indicate that the D at which fermentation starts depends upon the catabolite repression features of the strains under study (*Figures* 2–5). The latter is so despite the cells growing in glucose-limited chemostat cultures appear as catabolite repressed at all Ds. Our interpretation of these results is that the catabolite repression genotype would provide *S. cerevisiae* cells an enzymatic makeup and a pattern of metabolic fluxes that would determine at which growth rate yeast will start to ferment.

Our results do not contribute, however, to decide between mechanisms proposed to be involved in the triggering of fermentative behavior (see INTRODUCTION). In fact, given the behavior of q_{O_2} at various D, mechanisms encompassing limitation of the rate of transport of respiratory intermediates[3] or the overflow metabolism[4] would both fit well with the results obtained in this work.

The *snf1* mutant showed an extremely low yield that could not be justified by a reduced rate of respiration. *snf4* and *mig1* mutants displayed low P:O ratios, started fermentation at higher D, and did not exhibit such low Y_{Glc}. Furthermore, *snf4* and *mig1* strains were washed out at $D > 0.35\ h^{-1}$ whereas the *snf1* strain was at $D < 0.325\ h^{-1}$. The latter behavior indicated pleiotropic effects of the *snf1* deletion. Taken together, these results suggest that *SNF1* would not only be involved in the release of glucose-repressed gluconeogenic, glyoxylate cycle, respiratory genes or those implicated in the utilization of other carbon sources, but also in the regulation of the cellular energetic status. This is supported by the three- to fourfold lower ATP and ADP concentrations measured in extracts of *snf1* cells compared to those of the other strains tested in this work.[25]

Also the *snf1* mutant accumulated much larger amounts of intracellular G6P than the WT and other mutants.[25] The low ATP content of these cells may act as a driving force for a higher rate of electron transport at the level of the respiratory chain which was measured through the rate of oxygen consumption (*Figure 3*). Measurement of activities of glycolytic as well as gluconeogenic enzymes in yeast extracts did not support the idea that the energy-producing machinery may not be operative. In fact, we did not find in the *snf1* strain lower levels of glycolytic enzymes or increased futile cycling at the level of phosphofructokinase/fructose bisphosphatase as could be judged at least through their V_{max} values (not shown). Additionally, in the case of the *snf1* strain, we did not recover all the carbon consumed after analysis of biomass, ethanol, CO_2, glycerol, acetate, malate, and pyruvate. Although we have been able to recover over 93% of the carbon at all steady states in cultures with the WT, *snf4*, and *mig1* strains, we missed between 20–30% of the carbon in the case of the *snf1* mutant (*Table 3*). The redox balance indicated that a reduced compound should be excreted. This point deserves further study to fully characterize the effects of the *snf1* mutation and understand the functions of *SNF1* at physiological and cellular levels.

The *snf4* mutation has been shown to rescue the *tps1* (trehalose phosphate synthase) mutants inability to grow on glucose.[23] The rescue phenotype was similar to that of a *qcr1* gene that encodes a subunit of the bc1 complex or the treatment with respiratory inhibitors.[23] This is in agreement with the low P:O ratio (0.78) estimated for a *snf4* strain in our hands, and supports the involvement of glucose repression in triggering fermentative metabolism when a threshold q_{Glc} is attained.

Although Snf1 and Snf4 proteins appear to cooperate in the mechanism of expression of glucose repressible genes,[24] in the CEN.PK2 genetic background, the *snf4* mutant phenotype is less severe than that of *snf1*. The latter conclusion was reached through the following experimental evidence: i. The slow growth on maltose and raffinose (*Table 2*); ii. The intermediate critical dilution rate, D_c, exhibited in aerobic glucose-limited chemostat cultures (*Figure 4*); iii. The similarity of the physiological behavior with respect to the WT strain (*Figures 2* and *4*) (see also Cortassa and Aon[25]); and iv. The singular cell cycle behavior exhibited by the *snf4* strain both in batch and continuous cultures.[26] Such a behavior may be attributed to either a redundant protein in the CEN.PK2 genetic background that particularly overcomes the *snf4* defect and/or other functions of the Snf4 protein not directly associated with the mechanism of release from catabolite repression. A remarkable feature of strains on the CEN.PK2 background is that they produce large amounts of glycerol. In all cases, a high amount of carbon was recovered as glycerol even at low *D* when catabolism is oxidative. Around 10% of the carbon recovered appeared as glycerol in all strains assayed irrespective of the type of glucose breakdown displayed by yeast (*Table 3*). This behavior differs from that exhibited by other extensively characterized strains such as CBS 8066 which produces measurable levels of glycerol exclusively in the respirofermentative regime.[27]

The term "Crabtree effect" has been extensively used in the literature to design various phenomena.[21] The short-term Crabtree effect would in fact correspond to the original definition of Crabtree effect given to the phenomenon of "checking effect that tumor cells exert on their respiration" (reviewed in Cortassa and Aon[28] and Weusthuis *et al.*[2]). The so-called "long-term Crabtree effect" cannot indeed be explained by respiratory repression alone. Furthermore in this work, we provide evidence supporting the notion that the onset of fermentative metabolism in chemostat cultures is another feature of the complex phenomenon of glucose repression.

D Dilution rate
Dc Critical dilution rate

At low *D*, some yeast strains displayed respiratory metabolism and activities such as malate dehydrogenase or isocitrate lyase are derepressed whereas at high *D* the pattern of enzyme activities correspond to a repressed one[1] (reviewed in Aon and Cortassa).[7,8]

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