The Effect of Dissolved Oxygen Concentration on the Growth Physiology of *Saccharomyces cerevisiae* *whi2* Mutants

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(Received 12 October 1987; revised 28 April 1988)

Isogenic *whi2* and *WHI2*⁺ strains of *Saccharomyces cerevisiae* were grown in a 2-litre bioreactor as batch cultures on a medium containing yeast extract and peptone with either glucose or ethanol as carbon and energy source. The concentration of dissolved oxygen within the medium was varied over the range of 0 to 100% saturation. Expression of the *whi2* phenotype only occurred above 40% oxygen saturation with either glucose or ethanol as carbon and energy source. Under these conditions the *whi2* cells could be distinguished from *WHI2*⁺ cells in that they were phase dark, highly budded and very small during the stationary growth phase, and reached final cell densities four to six times higher than *WHI2*⁺ cells. The results clearly show that the *WHI2* gene of *S. cerevisiae* plays an important role in cell proliferation and that the availability of oxygen, or some product of oxidative metabolism, is involved in regulating the phenotypic expression of mutations within this gene.

INTRODUCTION

whi2 mutants of *Saccharomyces cerevisiae* lack the normal coordination of cell proliferation with nutrient availability (Sudbery *et al.*, 1980). Thus aerobic, batch cultivation of the parent (*WHI2*⁺) cells on YPG (yeast extract, 1%; peptone, 2%; glucose, 2%, w/v) medium results in cessation of cell proliferation at the onset of the stationary phase as a result of the exhaustion of the main carbon and energy source. Under these conditions the cell cycle arrests in the G1 phase with the majority of the cells in the unbudded state (Hartwell, 1974). By contrast, *whi2* cells continue to divide well into stationary phase and do not recognize the depletion of the carbon source. As a result of this division without cell growth the cells become smaller and attain a higher final cell concentration. When cell proliferation eventually ceases, presumably due to the exhaustion of the energy source or some other component of the medium, the cells arrest randomly within the cell cycle (Sudbery *et al.*, 1980).

A concerted series of physiological changes occurs with parent *WHI2*⁺ cells upon initiation of the stationary phase which results in distinct differences compared to exponentially growing cells. Stationary-phase cells are more resistant to a 52 °C heat shock (Parry *et al.*, 1976; Schenberg-Frascino & Moustacchi, 1972), to nystatin treatment (Snow, 1966) and to spheroplast formation (Deutch & Parry, 1973). In addition they appear phase bright (exponentially growing cells appear phase dark), and contain a higher proportion of glycogen and trehalose (Lillie & Pringle, 1980).

Abbreviation: dO₂, dissolved oxygen.

Under glucose-limited conditions, stationary-phase *whi2* cells retain the properties of exponentially growing cells and do not exhibit the physiological changes associated with wild-type cells (Sudbery *et al.*, 1980; Saul *et al.*, 1985). During growth on relatively poor carbon and energy sources such as glycerol, acetate and ethanol, *whi2* cells exhibit a specific growth rate much higher than that of *WHI2*⁺ cells (Walton, 1984).

These differences between the parent *WHI2*⁺ and mutant cells indicate that the *WHI2* gene plays an important role in the perception of the nutrient status of the environment or the ability of the cell to respond to changes in the environment in terms of cell size, growth rate and cell proliferation. The mechanism by which the *WHI2* gene effects or controls the physiological response of the cells is not known. Since the environment plays an important role in the expression of the *whi2* phenotype, we have examined the physiological differences between the mutant and parent under conditions where the dissolved oxygen concentration has been carefully controlled.

METHODS

Culture strains. The wild-type parent strain (*WHI2*⁺) of *S. cerevisiae* X4003-5B (a *ade1 his4 met2 ura3 trp5*) was obtained from the Yeast Stock Center, Berkeley, California, USA. The isogenic *whi2* mutant (ISO34) (α *whi2 his4 trp5 leu2*) has been previously described (Saul *et al.*, 1985).

Media and culture conditions. Cultures were grown on a YPG medium, containing Difco yeast extract (10 g l⁻¹), Difco Bacto-peptone (20 g l⁻¹) and glucose (20 g l⁻¹), at pH 6.5 and 30 °C, unless otherwise stated. When ethanol was used as the carbon and energy source the glucose was replaced by ethanol (10 g l⁻¹). Media were sterilized at 121 °C for 20 min with the exception of ethanol, which was filter-sterilized separately and added to the cooled, sterile medium.

All cultures were maintained on Petri dishes containing YPG medium plus Bacto-agar (Difco, 15 g l⁻¹) and were transferred monthly. Inocula for the 2-litre bioreactor were prepared by adding 100 ml of YPG, or YP-ethanol, medium to a 1-litre, cotton-wool-stoppered side-armed flask followed by inoculation with 3 ml of a culture previously grown on the same medium and originally obtained from a single colony. The flasks were incubated at 30 °C on a rotary shaker for 12–16 h, before using the whole contents as inoculum for the bioreactor.

A 2-litre (1.5 litre working volume) bioreactor (LSL Ltd) with temperature, pH and dissolved oxygen (dO₂) measurement and control was used for all batch culture experiments. dO₂ control was accomplished through PID-control (proportional-integral-differential control) of the agitator speed. Cultures in which the dO₂ was maintained above 40% saturation were supplied with 1 v.v.m. air (volume air per volume medium per minute). Below 40% saturation, nitrogen was supplied to the medium to lower the oxygen tension to the desired set-point value before inoculation. Air was then supplied at a rate of 0.2 v.v.m. except when the desired set-point was less than 10% saturation. The operating temperature was maintained constant at 30 °C.

After inoculation samples (22 ml) were removed from the bioreactor at intervals for the determination of cell and substrate concentration.

Determination of cell concentration. Cell concentration was determined in terms of optical density, cell dry weight (g l⁻¹) and total cell number (cells ml⁻¹).

Optical density measurements were determined at 600 nm using a Pye-Unicam SP 800 double-beam spectrophotometer against a water reference. Care was taken to dilute samples such that OD₆₀₀ measurements were directly proportional to cell number.

Cell dry weight was determined by centrifugation of culture samples (10 ml) at 15000 g for 10 min, washing the cell pellet with NaCl (9 g l⁻¹), re-centrifuging and placing the pre-weighed tubes containing cell pellet at 90 °C until the weight remained constant (15–18 h). Total cell number was determined microscopically using a graduated counting slide (haemocytometer).

Determination of cell size. This was done using a Coulter counter model ZB1, C1000 analyser and slave recorder which had been calibrated using latex beads (diameter 4.63 µm) as size standards.

Determination of glucose. The residual glucose content of the culture samples was determined by centrifugation (15000 g for 10 min) of the samples to remove cell material. The supernatant was then assayed enzymically using a glucose oxidase/peroxidase system coupled to the formation of 4-(*p*-benzoquinone-mono-imino)phenazone which was measured spectrophotometrically at 510 nm (Boehringer Mannheim automated analysis system 166391) and compared to a standard curve prepared with glucose (0–100 µg ml⁻¹).

Ethanol determination. Cell-free culture samples were injected into a gas chromatograph (Shimadzu model GC-Mini-1) fitted with a 2 m glass column containing Porapak Q as packing material. The column temperature was 125 °C with a carrier gas (N₂) flow rate of 30 ml min⁻¹. Acetone was used as internal standard.

RESULTS AND DISCUSSION

Batch culture growth on YPG

WHI2⁺ and *whi2* strains of *S. cerevisiae* were grown as batch cultures in the 2-litre bioreactor containing 1.5 l YPG medium plus 0.2 ml antifoaming agent (Sigma, Antifoam A) l⁻¹. The dO₂ concentration was maintained constant throughout each experiment by automatically controlling the agitation speed. The dO₂ concentration was measured using a sterilizable polarographic electrode which resulted in the dO₂ concentration varying from the set-point by no more than ±6%.

Typical batch growth curves for the mutant (*whi2*) grown at 0, 40 and >70% oxygen saturation of the medium are shown in Figs 1 (a), (b) and (c), respectively. Similar growth curves, with the exception of the total cell number, were obtained with the *WHI2*⁺ strain under similar conditions.

At low dO₂ concentrations (<40% saturation) both *WHI2*⁺ and *whi2* strains followed a basically anaerobic growth pattern in which cell dry weight and ethanol concentration increased, reaching a maximum 8–10 h after inoculation. The ethanol formed during this initial growth phase was not subsequently oxidized by the cells. The results (Table 1) showed no significant difference between the two strains in terms of specific growth rate (μ) (0.23–0.24 h⁻¹), final cell density (0.96 × 10⁸ cells ml⁻¹), final cell volume (34–36 μ m³), or the proportion of budded cells observed during the stationary phase (6.1–6.5%).

When the dO₂ was increased above 40% saturation, significant differences were observed between the two strains with respect to total cell number, cell volume and the percentage of budding cells observed in the stationary phase (Table 1). Thus at the onset of stationary phase the total cell number of the *whi2* strain (5.71–5.77 × 10⁸ ml⁻¹) was more than threefold higher than that of the *WHI2*⁺ strain (1.77–1.78 × 10⁸ ml⁻¹). However, the biomass yield coefficient, $Y_{x/s}$, for *whi2* was approximately 27% lower than for *WHI2*⁺ (Table 1), indicating that less of the substrate carbon had been conserved as biomass. Recent calorimetric experiments (unpublished

Table 1. Effect of dO₂ concentration on the growth and cell size distribution of strains X4003-5B (*WHI2*⁺) and ISO34 (*whi2*)

Strain genotype	Oxygen saturation (%)	μ (h ⁻¹)*	$Y_{x/s}$ †	10 ⁻⁸ × Final cell density (cells ml ⁻¹)‡	Final cell volume (μ m ³)	Budded cells (%)§
<i>WHI2</i>	0	0.23 (± 5%)	0.16 (± 1.8%)	0.96	34	6.2
	10	0.26 (± 4.8%)	0.19 (± 0.8%)	1.21	36	6.1
	20	0.21 (± 6.2%)	0.16 (± 1.2%)	1.50	35	3.8
	40	0.24 (± 3.2%)	0.16 (± 2.1%)	1.77	35	4.7
	50	ND	ND	ND	ND	ND
	> 70	0.23 (± 4.6%) [0.06 (± 5.2%)]	0.26 (± 1.6%)	1.78	30	6.5
<i>whi2</i>	0	0.24 (± 3.1%)	0.18 (± 1.2%)	0.96	36	6.8
	10	0.23 (± 4.2%)	0.17 (± 1.9%)	1.32	35	6.2
	20	0.23 (± 2.9%)	0.17 (± 1.3%)	1.72	36	5.9
	40	0.25 (± 3.8%)	0.15 (± 1.1%)	1.58	16–39	6.3
	50	0.30 (± 3.2%) [0.10 (± 4.6%)]	0.20 (± 1.3%)	5.77	18	26
	> 70	0.32 (± 3.8%) [0.11 (± 5.3%)]	0.18 (± 1.6%)	5.71	16	32

ND, Not determined.

* The results are shown ± the maximum percentage variation observed. Numbers in square brackets represent values for the subsequent growth phase on ethanol.

† Units: g cell dry weight formed (g glucose consumed)⁻¹. The results are shown ± the maximum percentage variation observed.

‡ Mean value determined from a minimum of five samples.

§ Figures represent the percentage of budded cells found during the stationary phase of growth from a total of 10³ cells counted.

Fig. 1. Batch culture growth of *S. cerevisiae* ISO34 (*whi2*) on YPG medium at different dO_2 concentrations: (a) 0%, (b) 40% and (c) >70% saturation of the medium with oxygen. ○, Cell dry weight; △, total number of cells ml^{-1} ; □, residual glucose concentration; ■, ethanol concentration.

data) suggest that the lower yield is due to a less efficient coupling of substrate oxidation to biosynthesis, with a considerable amount of the energy available within the substrate being lost as heat.

The result of a high cell number and low biomass yield is reflected in the small size of the *whi2* cells ($16\text{--}18\ \mu m^3$) compared with *WHI2*⁺ ($30\text{--}36\ \mu m^3$) (Fig. 2, Table 1).

A further major difference between the *WHI2*⁺ and *whi2* strains was the high degree of budding of the latter after exhaustion of the carbon source. Thus the *WHI2*⁺ cells occurred as individual or small clumps of unbudded cells, budded cells representing 3.8–6.5% of the total cell population, suggesting that upon exhaustion of the carbon source the cells arrest in the G1 stage of the cell cycle (Hartwell, 1974). The *whi2* cells appear to arrest randomly within the growth

Fig. 2. Cell size (volume, μm^3) distribution for *S. cerevisiae* ISO34 (*whi2*) during the stationary phase of batch growth on YPG medium at different dO_2 concentrations: (a) 0%, (b) 40%, (c) 50% and (d) >70% saturation of the medium with oxygen.

cycle (Sudbery *et al.*, 1980) and were consequently highly budded (26–32% of the total cell population).

At a dO_2 concentration of $40 \pm 6\%$ saturation, a situation midway between that for highly aerobic and anaerobic conditions was obtained (Table 1, and Fig. 2). The growth rate and final cell density for the *WHI2*⁺ and *whi2* strains were similar; however, two distinct peaks were observed for cell size distribution, the first ($16 \mu\text{m}^3$) characteristic of the *whi2* phenotype and the second ($39 \mu\text{m}^3$) characteristic of the *WHI2*⁺ phenotype (Fig. 2). It is interesting that no cells of intermediate phenotype were observed, suggesting that cells expressing the phenotype are in a discrete state, entry into which is controlled by a critical dO_2 level.

Batch culture growth on YP-ethanol

During batch growth on YPG medium, yeast cultures show diauxic growth. During the first phase cells consume glucose, which is metabolized fermentatively even in the presence of oxygen (Lagunas, 1986), and excrete ethanol. During the second phase the cells consume this ethanol, metabolizing it aerobically. *WHI2*⁺ and *whi2* cells are identical during the first phase

Table 2. *Physiological characteristics of strains X4003-5B (WHI2⁺) and ISO34 (whi2) grown aerobically on YP-ethanol medium*

Strain genotype	Oxygen saturation (%)	μ (h ⁻¹)	10 ⁻⁸ × Final cell density (cells ml ⁻¹)*	Final cell volume (μm ³)	Budded cells (%)†
WHI2 ⁺	>70	0.09	1.44	36	7.1
whi2	>70	0.16	7.82	12	35

* Mean value determined from a minimum of five samples.

† These figures represent the percentage of budded cells found during the stationary phase of growth from a total of 10³ cells counted.

Table 3. *Physiological characteristics of WHI2⁺ and whi2 strains after transfer from YPG medium at 0% O₂ saturation to >70% O₂ saturation*

	0% O ₂ saturation		>70% O ₂ saturation	
	WHI2 ⁺	whi2	WHI2 ⁺	whi2
μ (h ⁻¹)*	0.20 (± 3.1%)	0.26 (± 2.4%)	ND	ND
$Y_{N/S}$ * [g cell dry wt (g glucose) ⁻¹]	0.12 (± 1.6%)	0.15 (± 1.4%)	ND	ND
10 ⁻⁸ × Final cell density (cells ml ⁻¹)	0.69	1.38	1.52	7.07
Final cell volume (μm ³)	35	36	35	13
Budded cells (%)‡	4.8	6.1	5.4	24.6

ND, Not determined.

* The results are shown ± the maximum percentage variation observed.

† Mean value determined from a minimum of five samples.

‡ Figures represent the percentage of budded cells found during the stationary phase of growth from a total of 10³ cells counted.

but show differences in the stationary phase (Sudbery *et al.*, 1980). The requirement for a high degree of oxygen saturation to express the phenotype suggests that the phenotype is dependent on aerobic growth on ethanol. This conclusion is supported by the observation that a difference in cell size between the mutant and parent strain becomes apparent at the onset of ethanol consumption. This hypothesis was examined by growing cells on ethanol (10 g l⁻¹) as the major carbon and energy source. This concentration was chosen since it represents the maximum concentration obtained during growth on glucose. The dO₂ concentration was maintained above 70% saturation. The results (Table 2) show that final cell size, cell density and budding were characteristic of the *whi2* phenotype and similar to the final phenotype of cells grown in glucose batch cultures. Interestingly, the specific growth rate of the *whi2* strain on ethanol was 78% higher than that of the wild-type, confirming previous observations (Walton, 1984). Thus while *whi2* cells are identical to the wild-type during fermentative growth on glucose, they show marked differences during aerobic growth on ethanol.

Batch shift cultures grown on YPG

In order to further examine the role of oxygen in the expression of the *whi2* phenotype, a series of experiments was done in which WHI2⁺ and *whi2* strains were grown in the 2-litre bioreactor at dO₂ concentrations approaching 0% saturation. During the growth phase, samples (10 ml) were removed at intervals and placed in sterile flasks (100 ml) at 30 °C on a rotary shaker (250 r.p.m.), a speed which ensured >70% saturation of the medium with oxygen.

The results (Table 3) show that the *whi2* phenotype was not expressed during growth at low oxygen saturation. However, upon transfer to conditions of high oxygen saturation the final cell density, volume and percentage of budded cells were representative of the *whi2* phenotype. The results shown in Table 3 were independent of the time of sampling during the initial anaerobic growth period. Growth, during the initial anaerobic period, ceased after approximately 8 h due to glucose exhaustion. However, the *whi2* phenotype was expressed when samples taken during

this 8 h period, and up to 40 h after growth had ceased, were transferred to highly aerobic conditions. During the initial 8 h period, the residual glucose concentration in the medium decreased to zero as it was consumed by the cells for growth and ethanol formation. Consequently the samples transferred during this initial 8 h period contained a range of glucose and ethanol concentrations which appeared to have no effect on the degree of *whi2* phenotype expression.

The results obtained here with batch cultures agree with observations obtained with chemostat cultures. At high dilution rates the feed rate of glucose addition surpasses the ability of cells to metabolize it aerobically even in the presence of an adequate oxygen supply (Fiechter *et al.*, 1981; Sonnenleitner & Käppli, 1986). The result is an increase in fermentative activity of the yeast. As the dilution rate is decreased the availability of free glucose is decreased and the cells become derepressed. Cell size also changes with dilution rate (Lorincz & Carter, 1979; Johnston *et al.*, 1979; Lord & Wheals, 1980) decreasing with decreasing rate until it becomes independent of dilution rate at slow growth rates. The breakpoint of this curve is coincident with the dilution rate at the transition from fermentative to aerobic growth. However, it is not clear whether this is fortuitous (Walton, 1984). When *whi2* cells are cultured in a glucose-limited chemostat the breakpoint of the size/dilution rate curve is shifted towards a slower dilution rate. *whi2* cells are thus smaller than wild-type cells during aerobic growth but identical to wild-type cells during fermentative growth. Thus in both chemostat and batch culture studies the same conclusion is reached that expression of the *whi2* mutation requires aerobic growth. It thus seems likely that expression of the *whi2* phenotype requires a pattern of metabolism found during growth on non-fermentable carbon sources such as glycerol, ethanol and acetate (Walton, 1984). The exact significance of this observation is unclear; however, it suggests that examination of the mechanisms involved in catabolite repression, together with the enzymes involved in the control of metabolism, in *whi2* cells would further the understanding of this mutation.

Finally, it should be pointed out that the level of aerobicity of the cultures was controlled through automatic control of the rate of agitation of the bioreactor contents. This method was used since the mass transfer rate of oxygen from the gas to the liquid phase of the culture, and thus the dO₂ concentration, could be more accurately and rapidly controlled compared with other methods such as varying the air flow rate. It is theoretically possible that the results obtained for expression of the *whi2* phenotype were the result of a differential sensitivity of the parent and mutant strains to shear stress imposed by vigorous agitation. However, in view of the above discussion and the relatively low agitation rates employed (150–700 r.p.m.), this would appear unlikely.

This work was supported by an award from the Society of General Microbiology.

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