Antonie van Leeuwenhoek 67: 243–253, 1995.

Effects of growth conditions on mitochondrial morphology in *Saccharomyces cerevisiae*

Wiebe Visser[1], Edwin A. van Spronsen[2], Nanne Nanninga[2], Jack T. Pronk[1]*, J. Gijs Kuenen[1] & Johannes P. van Dijken[1]

[1] *Department of Microbiology and Enzymology, Kluyver Laboratory of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands;* [2] *Institute for Molecular Cell Biology, Biocentrum Amsterdam, Plantage Muidergracht 14, 1018 TV Amsterdam, The Netherlands (* requests for offprints)*

Received 15 July 1994; accepted in revised form 9 August 1994

Key words: mitochondria, morphology, *Saccharomyces cerevisiae*, vital staining, yeast

Abstract

Effects of growth conditions on mitochondrial morphology were studied in living *Saccharomyces cerevisiae* cells by vital staining with the fluorescent dye dimethyl-aminostyryl-methylpyridinium iodine (DASPMI), fluorescence microscopy, and confocal-scanning laser microscopy. Cells from respiratory, ethanol-grown batch cultures contained a large number of small mitochondria. Conversely, cells from glucose-grown batch cultures, in which metabolism was respiro-fermentative, contained small numbers of large, branched mitochondria. These changes did not significantly affect the fraction of the cellular volume occupied by the mitochondria. Similar differences in mitochondrial morphology were observed in glucose-limited chemostat cultures. In aerobic chemostat cultures, glucose metabolism was strictly respiratory and cells contained a large number of small mitochondria. Anaerobic, fermentative chemostat cultivation resulted in the large, branched mitochondrial structures also seen in glucose-grown batch cultures. Upon aeration of a previously anaerobic chemostat culture, the maximum respiratory capacity increased from 10 to 70 μmole.min^{-1}.g dry weight^{-1} within 10 h. This transition resulted in drastic changes of mitochondiral number, morphology and, consequently, mitochondrial surface area. These changes continued for several hours after the respiratory capacity had reached its maximum. Cyanide-insensitive oxygen consumption contributed ca. 50% of the total respiratory capacity in anaerobic cultures, but was virtually absent in aerobic cultures. The response of aerobic cultures to oxygen deprivation was qualitatively the reverse of the response of anaerobic cultures to aeration. The results indicate that mitochondrial morphology in *S. cerevisiae* is closely linked to the metabolic activity of this yeast: conditions that result in repression of respiratory enzymes generally lead to the mitochondrial morphology observed in anaerobically grown, fermenting cells.

Introduction

Yeasts are easy to grow under a variety of culture conditions and are therefore well suited for studies into the relation between structure and function of eukaryotic cells and organelles. The morphology of yeast mitochondria, the key organelles in respiratory metabolism, has been extensively studied over the past three decades (see e.g. Agar & Douglas 1957).

When *Saccharomyces cerevisiae*, a facultatively fermentative yeast, is grown under anaerobic conditions, its sugar metabolism is strictly fermentative. However, alcoholic fermentation also occurs under aerobic conditions when cells are exposed to excess sugar (Fiechter et al. 1981; Van Urk et al. 1988). Only when the supply of the sugar is growth limiting and the sugar concentration in the culture is thus poised at a low value, fully respiratory growth can be observed. Because of this dependence of respiratory metabolism on environmental conditions, a large number of studies have been performed on the effect of culture conditions on yeast mitochondrial morphology.

Electron microscopy is an excellent tool for studying subcellular organization. Yeast cells grown on non-

fermentable carbon sources clearly show mitochondrial structures (Marquardt 1962, 1963; Yotsuyanagi 1962). Under anaerobic growth conditions however, detection of mitochondria is more difficult, which has led to a dispute in the early literature as to whether or not anaerobically grown yeast cells contain mitochondria. This controversy has been resolved by the development of adequate staining and fixation techniques. It is now generally accepted that anaerobically grown yeast cells do contain mitochondria, sometimes called promitochondria (Schatz 1965; Damsky et al. 1969; Plattner & Schatz 1969; Plattner et al. 1971).

In the literature, apparent controversies exist with respect to the number of mitochondria per yeast cell and their morphology. Descriptions range from numerous ovoid-shaped mitochondria to a single branched mitochondrion and suggest that mitochondrial structure may be species dependent (Kawakami 1961; Prusso & Wells 1967; Hoffman & Avers 1973; Keddie & Barajas 1969). Hoffman & Avers (1973) showed that the many small mitochondria seen in thin-section electromicrographs may represent cross-sections of a single, branched organelle. Stevens (1977, 1981) reported that the mitochondrial number and morphology depended on the growth conditions.

Most experiments on mitochondrial morphology have been performed with batch cultures grown on glucose as a carbon and energy source. In such cultures, it is not possible to discriminate between effects of growth rate and glucose repression (van Dijken & Scheffers 1986; Alexander & Jeffries 1990). Furthermore, during cultivation in standard shake-flask cultures, oxygen limitation almost invariably occurs due to poor oxygen-transfer characteristics. The use of chemostat cultures makes it possible to control growth rate and dissolved-oxygen concentration and enables growth at low, derepressing glucose concentrations. The aim of the present study was to investigate the effect of environmental conditions on mitochondrial morphology in living *S. cerevisiae* cells grown in batch and chemostat cultures.

Although electron microscopy appears an obvious choice to study these morphological changes, it has a number of disadvantages, including the necessity of sample dehydration and the dependency of yeast-cell staining characteristics on growth conditions (Damsky et al. 1969). To circumvent these problems, we used the vital stain for mitochondria methylpyridinium iodine (DASPMI), a fluorescent, non-toxic stain for mitochondria (Bereiter-Hahn 1976; Bereiter-Hahn et al. 1983), combined with confocal scanning laser microscopy (CSLM) (Brakenhoff et al. 1979, 1985, 1989). CSLM allows the generation of high resolution, three-dimensional images of living cells, without the disadvantage of conventional fluorescence microscopy; i.e., the strong out-of-focus fluorescence light which reduces the contrast of images made at high magnifications.

Materials and methods

Microorganism and growth conditions

Diploid *Saccharomyces cerevisiae* was obtained by crossing the strains X2180-1A and X2180-1B (Yeast Genetic Stock Centre, Berkeley, California) and was maintained on malt-agar slopes.

Shake-flask cultivation

Repeated batch cultures were grown overnight at 28° C on an orbital shaker (200 rpm) in 250 ml erlenmeyer flasks containing 50 ml of a synthetic medium (Wickerham 1946) with either 4 $g.l^{-1}$ ethanol or 4 $g.l^{-1}$ glucose as a carbon and energy source. Cultures were grown for 8–10 generations until the OD_{450} read 0.2, at which time the exponentially growing cells were transferred to a new culture. Samples for microscopy were taken during exponential growth at an OD_{450} of approximately 0.2.

Chemostat cultivation

Chemostat cultivation was performed in 2-liter laboratory fermenters (Applikon Dependable Instruments b.v., Schiedam, the Netherlands) with a 1-liter working volume, at a dilution rate of 0.10 h^{-1}. The mineral medium, supplemented with vitamins, trace elements and the anaerobic growth factors ergosterol (5,7,22-ergostatrien-3β-ol; Sigma E-6510) and Tween-80 (polyoxyethylene-sorbitanmonooleate; Merck 822187) (see Andreasen & Stier 1953, 1954) was prepared as described by Weusthuis et al. (1993). Glucose was added to the sterile mineral medium after separate sterilization at 110° C. The pH was maintained at 5.0 by automatic addition of 2M KOH. The stirrer speed was maintained at 1000 rpm and the growth temperature was 30° C. The dissolved-oxygen concentration was measured with an Ingold autoclavable oxygen electrode. For anaerobic cultivation, the fermenter was flushed with 1 $l.min^{-1}$ pure nitrogen gas, containing

less than 5 ppm oxygen (Air Products, Waddinxveen, the Netherlands). The medium was made anaerobic before entering the culture by passing it through a second, sterile fermenter which was vigorously flushed with nitrogen. To minimize diffusion of oxygen, Norprene tubing (Cole-Parmer Instruments Corp., Chicago, USA) was used. For aerobic cultivation, the fermenter was sparged with air (1 $l.min^{-1}$).

Transient-state experiments

Transient-state experiments were performed by switching the gas supply of steady-state chemostat cultures from nitrogen to air or *vice versa*. This resulted in a rapid change in the dissolved-oxygen concentration: within 5 min, the dissolved-oxygen concentration had increased to above 30% air saturation or to below 0.1% air saturation, respectively. After the switch to aerobic conditions, the dissolved-oxygen concentration remained above 30% of air saturation throughout the experiment. Samples were collected from the effluent at appropriate time intervals and analysed for biomass dry weight, metabolite concentrations and mitochondrial morphology. Each transient-state experiment was performed in duplicate and showed good reproducibility. In this paper, data from two experiments are presented.

Analytical procedures

Culture dry weights were determined using a microwave oven and 0.45 μm membrane filters as described by Postma et al. (1989). Parallel samples varied by less than 1%. Concentrations of ethanol, glycerol and acetate were determined by HPLC (Weusthuis et al. 1993).

Oxygen-consumption measurements

The maximum respiratory capacity of culture samples was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, Ohio, USA) at 30° C. Cyanide-insensitive respiration was assayed in the presence of 1 mM KCN. Higher cyanide concentrations did not enhance the observed inhibitory effect.

Staining techniques for fluorescence microscopy

Cells were harvested by centrifugation (10 min at 4,000 × g) at room temperature, washed and resuspended in 0.1 M Tris-HCl buffer (pH 8.0). After 30 min incubation at room temperature with 10^6 M DASPMI, excess stain was removed by washing the cells with buffer.

Cell-volume measurements

Cell volumes (if not measured by CSLM; see below) were measured in an Olympus photomicroscope with a 100/1.3 lens. Images were collected with a video camera and transferred to a Macintosh IIci computer (Apple Computer Inc., Cupertino, California, USA). Measurements were made with a modified version of the program *Image* 1.35 (supplied by the Natl. Inst. of Health, Maryland) as described by Huls et al. (1992).

Conventional fluorescence microscopy

Conventional fluorescence microscopy was used when observing a large number of cells in a short period of time was more important than making three-dimensional reconstructions of a small number of cells, for instance during transient-state experiments and for determining the percentage of dividing cells. DASPMI-stained cells were placed between coverslips and observed in an Olympus photomicroscopy, equipped for epifluorescence with a 100-W high-pressure mercury arch lamp and a 100/1.3 lens. Filters for fluorescein-isothiocyanate (FITC) excitation were used. Photographs were made on Kodak TMAX-400 film and developed in Kodak D-76, thus pushing the sensitivity of the film to 1600 ASA.

Confocal-scanning laser microscopy (CSLM) techniques

For making optical sections, samples were photographed with a CSLM (prototype; University of Amsterdam), with its krypton ion laser (Spectra-Physics model 2020) tuned to 483 nm and a 510 nm blocking filter. In order to avoid rapid photobleaching, the output of the laser beam was kept constant at 10 mW and the illumination pinhole in the lightpath of the confocal microscope was kept as small as possible. To increase the signal-to-noise ratio, the detector pinhole of the microscope was slightly opened, thereby trading a small amount of confocality against a reduction of noise in the image. For the same reason 4 images per section were integrated, reducing this ratio further by a factor of 2. Yeast cells that were visualized in the CSLM were sectioned optically, usually in 16 lay-

ers, and stored as digital data on magnetic disk. After complete scanning of one section, the scanning stage containing the specimen was automatically raised by means of computer-controlled piezo-electric elements to prepare for the next section.

Image processing

In order to further decrease the amount of noise in the image, a 3-dimensional equivalent of a median filter was used on the images. The 3-D data were visualized by reprojection, a method simulating the optical characteristics (absorption, transmission, fluorescence) of these points, which are actually little volumes.

The success of this method critically depends on the signal-to-noise ratio of the image and the possibility of finding a threshold which separates objects from their surroundings. The suggestion of depth was enhanced by calculating the shadow which the object casts on an imaginary wall behind it. For a technical treatment of these aspects the reader is referred to Wu & Hesselink (1988) and Van der Voort et al. (1989).

Coulter counter

For electronic particle-counting, cells were lightly sonicated (1 s at 75 W output with a Branson sonifier) to avoid clumps. A 70 μm orifice tube (Coulter Electronics Ltd., Luton, England) was used at a current of 0.55 mA and with a solution of 0.9% (w/v) NaCl and 0.24% (v/v) formaldehyde as diluent.

Results

Mitochondrial morphology in repeated batch cultures growing on glucose or ethanol

For an initial comparison of mitochondrial structure in respiring and fermenting *S. cerevisiae*, confocal-scanning laser microscopy (CSLM) was performed on DASPMI-stained cells grown in aerobic repeated batch cultures on ethanol or glucose, respectively. This method ensures that the cells are continuously growing exponentially. The mitochondrial morphology in these cultures was very different: short, round or oval mitochondria were observed in ethanol-grown cells (Fig. 1A), whereas large, branched mitochondrial structures were observed in glucose-grown cells (Fig. 1B).

Light microscopy can be used for reliable measurement of the volume of large numbers of yeast cells (Huls et al. 1992). The cellular volume and the percentage of dividing cells in the ethanol- and glucose-grown cultures were determined by measuring about 250 cells. In a smaller number of cells (ca. 20), the number of mitochondria per cell and the mitochondrial volume were measured by CSLM optical sectioning. The most striking observation concerned the number of mitochondria, which was approximately ten-fold higher in ethanol-grown cells than in cells grown on glucose (Table 1). Although the number of mitochondria was different, the mitochondrial volume in ethanol- and glucose-grown cells was approximately the same (Table 1). This implies that mitochondria in ethanol-grown cultures have a much larger surface area than those in glucose-grown cells.

Glucose-limited chemostat cultures

Theoretically, the large mitochondrial surface area observed in ethanol-grown cells may be related to the high respiration rates in such cultures as compared to the glucose-grown batch cultures, in which respiratory enzymes are largely repressed. To eliminate influences of growth rate and glucose catabolite repression, the relation between respiratory capacity and mitochondrial morphology was further investigated in both aerobic and anaerobic glucose-limited chemostat cultures. The dilution rate was fixed at 0.10 h^{-1} to avoid the mixed respiro-fermentative metabolism that is exhibited by aerobic, sugar-limited chemostat cultures of *S. cerevisiae* at high dilution rates (von Meyenburg 1969; Postma et al. 1989).

In anaerobic, glucose-limited chemostat cultures, glucose metabolism was strictly fermentative. Under these conditions, all cells exhibited a mitochondrial morphology similar to that shown in Fig. 1B. In contrast, cells from aerobic glucose-limited chemostat cultures, in which glucose metabolism was strictly respiratory, contained many small mitochondria, similar to the morphology found with cells grown on ethanol in batch cultures (Fig. 1A).

The transition from anaerobic, fermentative growth to aerobic, respiratory growth results in drastic changes in the metabolism of *S. cerevisiae*. To investigate these changes and their impact on mitochondrial morphology, transient-state experiments were performed, in which anaerobic chemostat cultures were switched to aerobic conditions and *vice versa*.

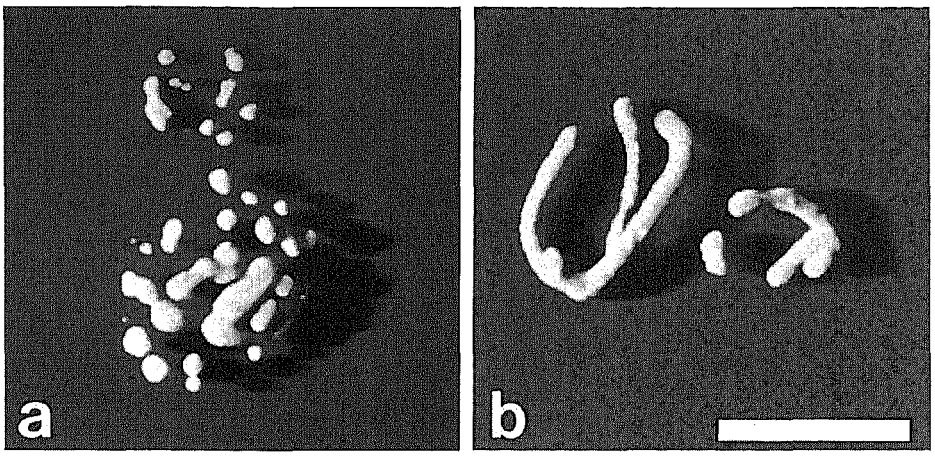

Fig. 1. Reprojected (Wu & Hesselink 1988; van der Voort et al. 1988) CSLM images of the mitochondria of budding *S. cerevisiae* cells grown in repeated batch cultures with ethanol as a non-fermentable carbon source (A) and with a relatively high concentration of glucose (B). Bar = 5 μm.

Table 1. Effect of growth substrate on morphology of *S. cerevisiae* cells and mitochondria. Exponentially growing cells were sampled from repeated batch cultures grown on the fermentable substrate glucose or the non-fermentable substrate ethanol.

Growth substrate	Growth phase	Cells			Mitochondria	
		%[1]	volume[2]	N[3]	Number per cell[4]	Relative volume[5]
Glucose	non-budding	48	55	9	2.3	7.4
Glucose	budding	52	79	13	3.2	7.4
Ethanol	non-budding	53	43	11	20–30	6.3
Ethanol	budding	47	63	10	20–30	6.2

[1] The percentage of (non)-budding cells was calculated from ca. 250 measurements with an ordinary light microscope.

[2] The average volume (μm^3) was calculated from ca. 250 measurements with an ordinary light microscope.

[3] Number of cells analyzed by CSLM.

[4] Average number of mitochondria per cell as measured by CSLM optical sectioning.

[5] Relative volume of mitochondria as percentage of cell volume.

Table 2. Changes in cell size, biomass concentration and cell mass during a switch from anaerobic to aerobic conditions in a glucose-limited chemostat culture. Cell-size measurements were performed with a Coulter counter (see Materials and methods).

Time (h)	Average size (% of initial value)	Dry weight ($g.l^{-1}$)	Cell number (ml^{-1})	Cellular mass (mg dry weight.$cell^{-1}$)
0	100	1.27	56.10^6	2.310^{-11}
2	112	1.40	78.10^6	1.810^{-11}
25	140	6.16	293.10^6	2.110^{-11}

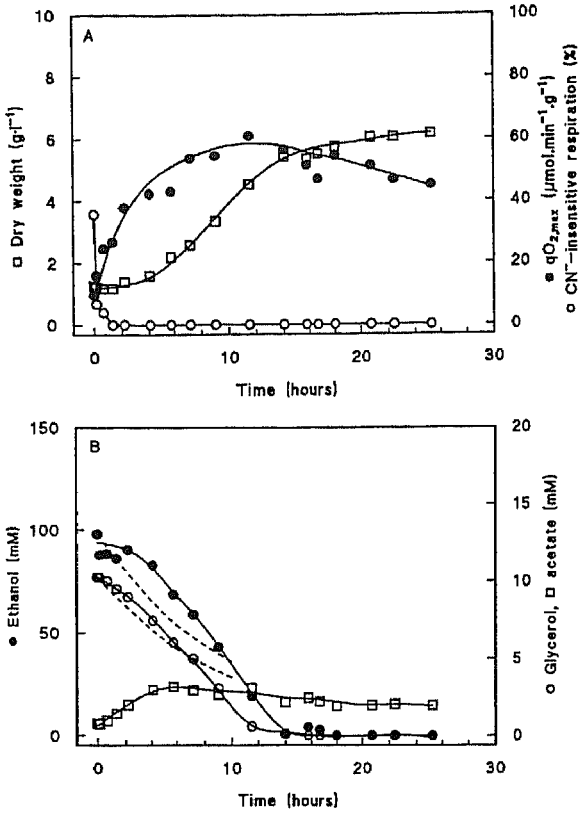

Fig. 2. Transient response of an anaerobic, glucose-limited chemostat culture of *S. cerevisiae* after a shift to aerobic conditions. A: Biomass concentration, maximum respiratory capacity and contribution of cyanide-insensitive respiration to the maximum respiratory capacity. B: Concentrations of ethanol, glycerol and acetate. The dashed lines show wash-out kinetics (no net consumption or production) for glycerol and ethanol. Growth conditions: $D = 0.10\ h^{-1}$, pH 5.0, T = 30° C, reservoir concentration of glucose 11.5 $g.l^{-1}$.

Transition from anaerobic to aerobic conditions

In aerobic and anaerobic steady-state chemostat cultures, the specific growth rates are equal to the dilution rate. However, the biomass yield of *S. cerevisiae* on glucose in aerobic, sugar-limited cultures is approximately five-fold higher than in anaerobic cultures (Verduyn 1991). This difference, which is due to the contribution of oxidative phosphorylation to energy transduction, implies that the biomass concentration will increase during a switch from anaerobic to aerobic conditions and, consequently, that the growth rate will increase during the transient state. The expected increase in biomass concentration was indeed observed: during the transition from anaerobic to aerobic growth, the growth rate transiently increased to 0.2–0.3 h^{-1} (Fig. 2A). Although the dry weight per cell appeared to be fairly constant during the transition, the cell volume increased by ca. 40%, suggesting that the water content of the cells increased (Table 2).

After shifting an anaerobic, fermentative culture to aerobic conditions, the culture can still produce ATP from fermentative metabolism, but also from respiration of glucose, ethanol, glycerol and other fermentation products. To follow the change from fermentative to respiratory metabolism, concentrations of relevant metabolites were measured in the fermenter effluent. In chemostat cultures, the concentration of metabolites is the net result of production, consumption and dilution (wash-out). Immediately after addition of oxygen to the culture, the concentrations of ethanol and glycerol in the culture medium decreased (Fig. 2B). However, during the first two hours after the shift, the decrease of the glycerol concentration was slower than calculated on the basis of wash-out kinetics (Fig. 2B). Apparently, glycerol formation continued during this period, albeit at a lower rate than in the anaerobic culture. Similarly, alcoholic fermentation appeared to continue for approximately 4 h after the shift. After this period, utilization of ethanol and glycerol occurred (Fig. 2B).

Maximum respiratory capacities (qO_2^{max}) increased rapidly after switching from anaerobic to aerobic conditions (Fig. 2A), reaching a maximum approximately 8 h after the switch. It is well known that alternative, cyanide-insensitive respiratory systems are often expressed in yeasts when normal respiration is limited (Alexander & Jeffries 1990). Indeed, cyanide-insensitive oxygen consumption made up approximately half of the total respiratory capacity in anaerobically grown cells (Fig. 2A). Upon the switch from anaerobic to aerobic conditions, cyanide-insensitive respiratory capacity decreased very sharply during the first hour, and was virtually absent in cells from aerobic cultures (less than 1 $\mu mol.min^{-1}.g^{-1}$; Fig. 2A).

During the shift from anaerobic to aerobic conditions, mitochondrial morphology was regularly studied by fluorescence microscopy (Fig. 3). The various stages show that the change in mitochondrial morphology became evident about 8 h after the shift to aerobic conditions. Since these transient states were performed in continuous cultures, cells were growing and being washed out continuously, giving rise to a partially new population. Nevertheless, the cultures remained homogeneous with respect to mitochondrial structure throughout the transient-state experiments

(data not shown). This indicates mitochondrial morphology was changing within existing cells, rather than by the appearance of daughter cells with a different mitochondrial structure.

A remarkable difference with respect to time scale was observed when the changes in respiratory capacity and in mitochondrial morphology were compared (Figs 2A and 3). After 7 h the cells still contained large, branched mitochondria, although the respiratory capacity was already near its maximum.

Transition from aerobic to anaerobic conditions

When an aerobic chemostat culture is shifted to anaerobic conditions, respiration ceases as a result of the absence of oxygen and energy transduction becomes critically dependent on fermentation (i.e. substrate-level phosphorylation). The lower ATP yield of alcoholic fermentation was expected to lead to a temporary reduction of the growth rate. Indeed, microscopy revealed a decrease in the percentage of budding cells (data not shown). Furthermore, the biomass concentration decreased during the first hours due to washout (Fig. 4A). After the shift to anaerobiosis, ethanol appeared virtually immediately (Fig. 4B). Also glycerol formation set in immediately after the shift. Glycerol is produced by *S. cerevisiae* when the surplus NADH that is formed during biomass synthesis cannot be reoxidized by respiration (Weusthuis et al. 1994).

When aerobic cells were deprived of oxygen, a rapid decrease of the oxygen-uptake capacity was observed during the first hours (Fig. 4A). The capacity of cyanide-insensitive oxygen consumption increased from 1 to 7 μmol.min^{-1}.g^{-1} and eventually, in the anaerobic steady-state culture, contributed 50% of the total oxygen consumption.

Fluorescence microscopy of DASPMI-stained samples taken during the switch to anaerobic conditions demonstrated that the adaptation of mitochondrial morphology shown in Fig. 3 was reversible. The many small mitochondria that were observed in aerobic cultures evolved into a few, large and branched structures over a period of approximately 10 h (data not shown).

Discussion

Most studies on the effect of environmental conditions on yeast mitochondria have focused on the expression of key respiratory enzyme activities, including TCA-cycle enzymes and components of the respiratory chain. In *S. cerevisiae*, regulation of the expression of many of these enzymes has been studied in detail at the molecular level. It is generally dependent on the presence of oxygen and subject to glucose catabolite repression (for a review see De Winde & Grivell 1993).

Investigations into the relation between environmental conditions and mitochondrial morphology has yielded conflicting results. This may be due to technical problems related to visualization of (pro)mitochondria and the maintenance of well-defined growth conditions. Many studies have been performed under poorly defined growth conditions in shake-flask cultures, which implies that conditions that are of key importance in the regulation of mitochondrial activity (in particular glucose-concentration and dissolved-oxygen concentration) cannot be controlled. Perhaps for this reasons, clear correlations between environmental conditions and mitochondrial number, volume and shape were often not observed.

In the present study, low-cell density repeated batch cultivation and chemostat cultivation have been used to avoid some of the problems inherent to the use of standard shake-flask cultures. Vital staining of mitochondria with the fluorescent dye DASPMI, in combination with confocal-scanning laser microscopy (CSLM) obviated the use of the fixation and dehydration steps that are required for electron microscopy.

The extreme situations that were encountered with respect to mitochondrial morphology are illustrated in Fig. 1. The exclusively respiratory metabolism in ethanol-grown cells was accompanied by the presence of many small mitochondria. A similar morphology was observed in aerobic, glucose-limited chemostat cultures. One or a few large, branched mitochondria were observed in cells from exponentially growing, aerobic batch cultures on glucose, in which metabolism is predominantly fermentative due to glucose repression of respiratory enzymes (De Winde & Grivell 1993; Alexander & Jeffries 1990). This morphology was also observed in cells from anaerobic, glucose-limited chemostat cultures. These differences in mitochondrial morphology were also readily apparent with conventional fluorescence microscopy, which was therefore used to study mitochondrial morphology during transient-state experiments in chemostat cultures.

Our data indicate that, similar to the regulation of the key enzymes of mitochondrial respiration, the morphology of *S. cerevisiae* mitochondria is influenced

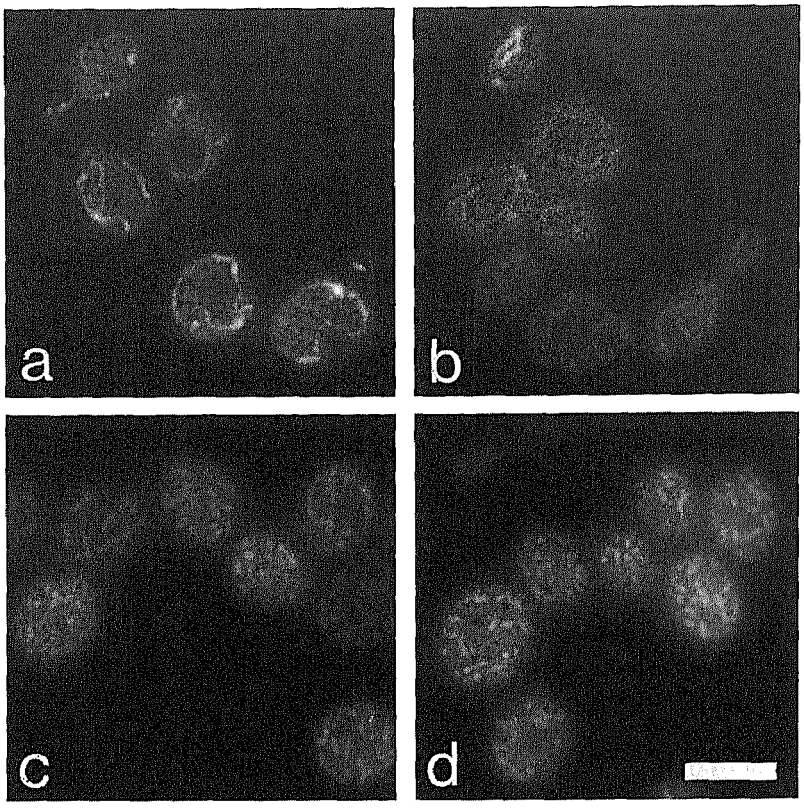

Fig. 3. Fluorescence microscopy of DASPMI-stained mitochondria in *S. cerevisiae*. Photographs were taken at various time intervals after a shift from anaerobic to aerobic growth conditions in a sugar-limited chemostat culture: A, anaerobic steady-state culture; B, 7 h after the shift; C, 11.5 h after the shift; D, 18 h after the shift: at this stage the morphology was indistinguishable from that of an aerobic steady-state culture. Growth conditions as in Fig. 2.

Table 3. Mitochondrial morphology under different cultivation conditions as observed with fluorescence microscopy of DASPMI-stained cells. Substrate excess refers to cells grown in batch cultures on 4 $g.l^{-1}$ carbon source. Substrate-limited conditions refer to chemostat cultures ($D = 0.10\ h^{-1}$) fed with a medium in which the carbon source was the growth-limiting factor. The glucose concentration in such cultures was below 100 $mg.l^{-1}$. Absence of oxygen refers to a dissolved oxygen concentration below the detection limit of commercially available oxygen probes. The terms repressed/derepressed refer to well-documented (Gancedo & Serrano 1989) regulation of various TCA-cycle and respiratory enzymes. Mitochondrial morphology refers to the differences shown in Fig. 1.

Growth substrate	Concentration	Oxygen	Respiratory enzymes	Mitochondrial morphology
glucose	excess	present	repressed	few, large
ethanol	excess	present	derepressed	many, small
glucose	excess	absent	repressed	few, large
glucose	limiting	absent	repressed	few, large
glucose	limiting	present	derepressed	many, small

by a combination of oxygen availability and glucose concentration (Table 3). Situations that are known to lead to repression of respiratory enzymes (anaerobic conditions and/or presence of excess glucose) were invariably accompanied by the presence of one or a few large, branched mitochondria per cell. In contrast, many small mitochondria were observed in cells grown under derepressing conditions (aerobic growth on ethanol or in aerobic, glucose-limited chemostat cultures). The relation between mitochondrial morphology and growth conditions observed in the present study is generally in compliance with that observed by Stevens (1977, 1981), who studied mitochondrial morphology by electron microscopy and serial sectioning. A strikingly different pattern has been reported by Gélinas & Goulet (1991). Using freeze-etching techniques, these authors found large, branched mitochondria in highly aerated, fed-batch grown cells and small mitochondria in cells lacking oxygen. In our study, the different mitochondrial number and morphology observed in ethanol- and glucose-grown cells did not significantly affect the fraction of the cellular volume occupied by the mitochondria (6–7%, Table 1). In contrast, Stevens (1977) observed that mitochondrial volumes for glucose-grown cells and cells grown on a nonfermentable carbon source were different (3% and 13%, respectively). The mitochondrial volumes found in the present study were ca. 50% lower than those observed by Grimes et al. (1974).

With a constant mitochondrial volume, a change in morphology from a single, large mitochondrion to a large number of small organelles leads to an increase of the total mitochondrial surface area. This might reflect an increased membrane-area requirement, necessary to accommodate newly synthesized components of the respiratory chain. However, during a shift from anaerobic to aerobic conditions, changes in mitochondrial morphology continued for several hours after the respiratory capacity had reached its maximum (Figs 2 and 3). This indicates that presence of many small mitochondria is not a prerequisite for the increased respiration rate. In this respect, it is important to bear in mind that the membrane area available for respiratory enzymes is also dependent on the fine-structure of the mitochondrial inner membrane, which starts developing when cells are shifted to aerobic conditions (Damsky et al. 1976).

The present study indicates that chemostat cultivation, in combination with vital staining, is a useful tool for studies into the relation between growth conditions and mitochondrial morphology. This method

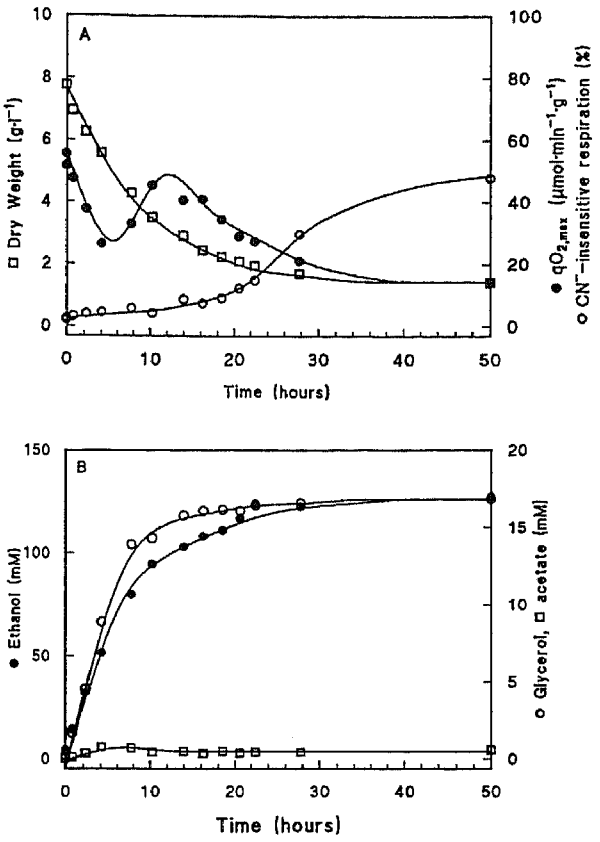

Fig. 4. Transient response of an anaerobic, glucose-limited chemostat culture of *S. cerevisiae* after a shift to aerobic conditions. A. Biomass concentration, maximum respiratory capacity and contribution of cyanide-insensitive respiration to the maximum respiratory capacity. B: Concentrations of ethanol, glycerol and acetate. Growth conditions: $D = 0.10\ h^{-1}$, pH 5.0, T = 30° C, reservoir concentration of glucose $14.6\ g.l^{-1}$.

can be extended to other situations that are particularly interesting with respect to the regulation of respiratory capacity in yeasts. A number of options are available to manipulate respiratory activity in yeasts, including aerobic, glucose-limited growth at high dilution rates, which leads to a mixed respiro-fermentative metabolism (Von Meyenburg 1969; Postma et al. 1989), growth under a double limitation of glucose and oxygen (Weusthuis et al. 1994) and growth in the presence of uncoupling weak acids, which leads to very high respiration rates and an increase of the mitochondrial volume (Verduyn et al. 1992). In view of the conflicting results with some earlier reports and in order to obtain more detailed information on mitochondrial structure, in particular with respect to inner-membrane surface area (Damsky et al. 1976), it is

essential that such work should also include the use of electron microscopic techniques.

Acknowledgements

These investigations were supported by the Foundation for Biological Research (BION), which is subsidized by the Netherlands Organization for Scientific Research (NWO). DASPMI was a gift from Prof. J. Bereiter-Hahn (J.W. Goethe Universität, Frankfurt).

References

Agar HD & Douglas HC (1957) Studies on the cytological structure of yeast: electron microscopy of thin sections. J. Bact. 73: 365–375.

Alexander MA & Jeffries TW (1990) Respiratory efficiency and metabolite partitioning as regulatory phenomena in yeasts. Enzyme Microb. Technol. 12: 2–19.

Andreasen AA & Stier TJB (1953) Anaerobic nutrition of *S. cerevisiae*. I. Ergosterol requirement for growth in a defined medium. J. Cell. Comp. Physiol. 41: 23–26.

— (1954) Anaerobic nutrition of *S. cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. J. Cell. Comp. Physiol. 43: 271–281.

Bereiter-Hahn J (1976) Dimethylaminostyrylmethylpyridiniumiodine (DASPMI) as a fluorescent probe for mitochondria *in situ*. Biochim. Biophys. Acta 423: 1–14.

Bereiter-Hahn J, Seipel KH, Vöth M & Ploem JS (1983) Fluorometry of mitochondria in cells vitally stained with DASPMI or rhodamine 6 GO. Cell Biochem. and Function 1: 147–155.

Brakenhoff GJ, Blom P & Barends PJ (1979) Confocal scanning light microscopy with high aperture immersion lenses. J. Microsc. 117: 219–232.

Brakenhoff GJ, van der Voort HTM, van Spronsen EA, Linnemans WAM & Nanninga N (1985) Three-dimensional chromatin distribution in neuroblastoma nuclei shown by confocal scanning laser microscopy. Nature 317: 748–749.

Brakenhof GJ, van Spronsen EA, van der Voort HTM & Nanninga N (1989) Three-dimensional confocal fluorescence microscopy. Meth. Cell Biol. 30: 379–398.

Damsky CH, Nelson WM & Claude A (1969) Mitochondria in anaerobically-grown, lipid-limited brewer's yeast. J. Cell Biol. 43: 174–179.

Damsky CH (1976) Environmentally induced changes in mitochondria and endoplasmic reticulum of *Saccharomyces carlsbergensis* yeast. J. Cell Biol. 71: 123–135.

De Winde JH & Grivell LA (1993) Global regulation of mitochondrial biosynthesis in *Saccharomyces cerevisiae*. Progr. Nucleic Acids Res. Mol. Biol. 46: 51–91.

Fiechter A, Furhmann GF & Käppeli O (1981) Regulation of glucose metabolism in growing yeast cells. Adv. Microbial Physiol. 22: 123–183.

Gancedo C & Serrano R (1989) Energy-yielding metabolism. In: Rose AH & Harrison JS (Eds). The molecular biology of the yeast *Saccharomyces*. Metabolism and gene expression. Cold Spring Harbor, New York, pp 1–37.

Gélinas P & Goulet J (1991) Morphology of bakers' yeast and dissolved oxygen saturation during fed-batch growth. Lett. Appl. Microbiol. 12: 164–170.

Grimes GW, Mahler HR & Perlman PS (1974) Nuclear gene dosage effects on mitochondrial mass and DNA. J. Cell Biol. 61: 565–574.

Hoffmann HP & Avers CJ (1973) Mitochondrion of yeast: ultrastructural evidence for one giant, branched organelle per cell. Science 181: 749–751.

Huls PG, Nanninga N, van Spronsen EA, Valkenburg JAC, Visscher NOE & Woldringh CL (1992) A computer supported measuring system for the characterization of yeast populations combining 2D-image analysis, Coulter counter and flow cytometry. Biotechn. Bioeng. 39: 343–350.

Kawakami N (1961) Thread-like mitochondria in yeast cells. Expl. Cell Res. 25: 179–181.

Keddie MK & Barajas L (1969) Three-dimensional reconstruction of *Pityrosporum* yeast cells based on serial section electron microscopy. J. Ultrastruc. Res. 29: 260–275.

Marquardt H (1962) Der Feinbau von Hefezellen im Elektronenmikroskop. II. Mitt.: *Saccharomyces cerevisiae* – stämme. Z. Naturf. 17B: 689–695.

— (1963) Elektronenoptische Untersuchungen über die Ascosporenbildung bei *Saccharomyces cerevisiae* unter cytologischem und cytogenetischem Aspekt. Arch. Mikrobiol. 46: 308–320.

Plattner H & Schatz G (1969) Promitochondria of anaerobically-grown yeast. III. Morphology. Biochemistry 8: 339–343.

Plattner H, Salpeter M, Saltzgaber J, Rouslin W & Schatz G (1971) Pro-mitochondria of anaerobically-grown yeast: evidence for their conversion into functional mitochondria during respiratory adaptation. In: Boardman NK, Linnane AW & Smillie RM (Eds). Autonomy and biogenesis of mitochondria and chloroplasts (pp 175–184). North-Holland Publishing Company, Amsterdam.

Postma E, Verduyn C, Scheffers WA & van Dijken JP (1989) Enzymatic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 55: 468–477.

Prusso DC & Wells K (1967) *Sporobolomyces roseus*. I. Ultrastructure. Mycologia 59: 337–348.

Schatz G (1965) Subcellular particles carrying mitochondrial enzymes in anaerobically-grown cells of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 96: 342–345.

Stevens BJ (1977) Variation in number and volume of the mitochondria in yeast according to growth conditions. A study based on serial sectioning and computer graphics reconstitution. Biol. Cellulaire 28: 37–56.

Stevens BJ (1981) Mitochondrial Structure. In: Strathers JS, Jones EW & Broach JR (Eds). The Molecular Biology of the Yeast *Saccharomyces* – Life Cycle and Inheritance (pp 471–504). Cold Spring Harbor, New York.

Van der Voort HTM, Brakenhoff GJ & Baarslag MW (1989) Three-dimensional visualization methods for confocal microscopy. J. Microsc. 153: 123–132.

Van Dijken JP & Scheffers WA (1986) Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol. Rev. 32: 199–224.

Van Urk H, Mak PR, Scheffers WA & van Dijken JP (1988) Metabolic responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon transition from glucose limitation to glucose excess. Yeast 4: 283–291.

Verduyn C (1991) Physiology of yeasts in relation to growth yields. Antonie van Leeuwenhoek 60: 325–353.

Verduyn C, Postma E, Scheffers WA & van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeast: a continuous-culture

study on the regulation of respiration and alcoholic fermentation. Yeast 8: 501–517.

Von Meyenburg HK (1969) Energetics of the budding cycle of *Saccharomyces cerevisiae* during glucose limited aerobic growth. Arch. Microbiol. 66: 289–303.

Weusthuis RA, Adams H, Scheffers WA & van Dijken JP (1993) Energetics and kinetics of maltose transport in *Saccharomyces cerevisiae*: a continuous-culture study. Appl. Environ. Microbiol. 59: 3102–3109.

Weusthuis RA, Visser W, Pronk JT, Scheffers WA & van Dijken JP (1994) Effects of oxygen limitation on sugar metabolism in yeasts: a continuous-culture study of the Kluyver effect. Microbiology 140: 703–715.

Wickerham LJ (1946) A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. J. Bacteriol. 52: 293–301.

Wu K & Hesselink L (1988) Computer display of reconstructed 3-D scalar data. Appl. Optics 27: 395–404.

Yotsuyanagi Y (1962) Etudes sur la chondriome de la levure. I. Variation de l'ultrastructure du chondriome au cours du cycle de la croissance aérobie. J. Ultrastructure. Res. 7: 121–140.