Cycles of mitochondrial energization driven by the ultradian clock in a continuous culture of *Saccharomyces cerevisiae*

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A continuous culture of *Saccharomyces cerevisiae* IFO 0233, growing with glucose as the major carbon and energy source, shows oscillations of respiration with a period of 48 min. Samples taken at maxima and minima indicate that (i) periodic changes do not occur as a result of carbon depletion, (ii) intrinsic differences in respiratory activity occur in washed organisms and (iii) a respiratory inhibitor accumulates during respiratory oscillations. Plasma membrane and inner mitochondrial membranes generate transmembrane electrochemical potentials; changes in these can be respectively assessed using anionic or cationic fluorophores. Thus flow cytometric analyses indicated that an oxonol dye [DiBAC₄(3); bis(1,3-dibutylbarbituric acid)trimethine oxonol] was excluded from yeasts to a similar extent (in >98% of the population) at all stages, showing that the plasma membrane potential was maintained at a steady value. However, uptake of Rhodamine 123 was greatest at that phase characterized by a low respiratory rate. Addition of uncouplers of energy conservation [CCCP (*m*-chlorocarbonylcyanide phenylhydrazone) or S-13 (5-chloro-3-*t*-butyl-2-chloro-4'-nitrosalicylanilide)] to the continuous cultures increased the respiration, but had only a transient effect on the period of the oscillation. Electron microscopy showed changes in mitochondrial ultrastructure during the respiratory oscillation. At low respiration the cristae were more clearly defined due to swelling of the matrix; this corresponds to the 'orthodox' conformation. When respiration was high the mitochondrial configuration was 'condensed'. It has been shown previously that a temperature-compensated ultradian clock operates in *S. cerevisiae*. It is proposed that mitochondria undergo cycles of energization in response to energetic demands driven by this ultradian clock output.

Keywords: mitochondria, redox regulation, NADH, cytochrome, ultradian clock

INTRODUCTION

Continuous cultures of *Saccharomyces cerevisiae* display a temperature-compensated rhythm of respiration which can be continuously monitored over extended periods (months) by measurement of dissolved O₂ (Satroutdinov *et al.*, 1992). This is one output of many

driven by the ultradian clock (Murray *et al.*, 2001), which has, in this yeast strain, a characteristic period of about 40 min over the range 25–35 °C (temperature quotient; $Q_{10}=1.07$). Other cyclically varying outputs include sulphate uptake (Sohn & Kuriyama, 2001), ethanol production (Keulers *et al.*, 1996a), glutathione to oxidized glutathione dimer interconversion (Murray *et al.*, 1999) as well as intracellular redox states, as indicated by continuous *in vivo* monitoring of NADH fluorescence (Murray *et al.*, 1998a, b), and calculated values of intracellular pH (Satroutdinov *et al.*, 1992). It is proposed under the highly acid culture conditions employed that metabolic synchrony is elicited by per-

Abbreviations: CCCP, *m*-chlorocarbonylcyanide phenylhydrazone; DiBAC₄(3), bis(1,3-dibutylbarbituric acid)trimethine oxonol; S-13, 5-chloro-3-*t*-butyl-2-chloro-4'-nitrosalicylanilide; DOT, dissolved O₂ tension; SHAM, salicylhydroxamic acid.

Fig. 1. Oscillatory outputs of the ultradian clock in a continuous culture of *S. cerevisiae*. (a) DOT (dashed line), NADH fluorescence (continuous trace), both measured directly, simultaneously and continuously, and ethanol concentration ($\circ$). (b) DOT (dashed line), acetaldehyde ($\blacktriangle$) and H₂S ($\triangle$).

iodic release of H₂S, 180° out of phase with acetaldehyde accumulation (Sohn *et al.*, 2000).

The oscillatory behaviour of respiration is not dependent on glycolytic oscillations as is evidenced by its occurrence with a similar persistence and temperature-compensated period during growth with ethanol (Keulers *et al.*, 1996a, b; Murray *et al.*, 2001). Glycolytic oscillations are different in that they are characterized by shorter periods (about 1 to 5 min), are highly damped and not temperature-compensated (Lloyd & Edwards, 1984, 1987).

The aim of the present work is to study changes in mitochondrial structure and function *in situ* during respiratory oscillations. We conclude that growth is accompanied by *in vivo* respiratory control (ADP-acceptor control) involving cycles of energization and de-energization of mitochondria (Chance & Williams, 1956; Luzikov, 1984; Lloyd, 1974) in response to energetic demand.

METHODS

Organisms and cultures. *Saccharomyces cerevisiae* strain IFO 0233 was used (Keulers *et al.*, 1996a). The medium consisted of glucose monohydrate (22 g l⁻¹), (NH₄)₂SO₄ (5 g l⁻¹), KH₂PO₄ (2 g l⁻¹), MgSO₄·7H₂O (0.5 g l⁻¹), CaSO₄·5H₂O

(0.005 g l⁻¹), MnCl₂·4H₂O (0.001 g l⁻¹), yeast extract (Difco; 1 g l⁻¹) and 70% (v/v) H₂SO₄ (1 ml l⁻¹). Sigma Antifoam A was used at 1 ml l⁻¹.

Continuous culture was carried out as described previously (Satroutdinov *et al.*, 1992). The fermenter (LH500; Adaptive Biosystems) was operated with a stirrer rate of 900 r.p.m., a working volume of 0.8 l, an airflow rate of 180 ml min⁻¹ and a dilution rate of 0.085 h⁻¹. The temperature was 30 °C and the pH was controlled at 3.4 by the addition of 2.5 M NaOH. Dissolved O₂ was monitored continuously using an Ingold O₂ electrode. Experiments were conducted in continuous light. On line data acquisition was carried out using an in-house constructed PC-based system. Uncouplers of energy conservation [CCCP (*m*-chlorocarbonylcyanide phenylhydrazine) and S-13 (5-chloro-3-*t*-butyl-2-chloro-4'-nitrosalicylanilide)] were added as alcoholic solutions.

Cellular respiration. Organisms in growth medium were transferred directly to a closed O₂ electrode system at 30 °C (Rank) for measurements of respiration of the culture samples (2 ml) taken at maxima and minima of dissolved O₂ tension (DOT) in the oscillating state. Inhibitors [Na azide, SHAM (salicylhydroxamic acid)], or uncouplers (CCCP, S-13) (Hanstein, 1976) were added during the initial linear stage of O₂ consumption. Where washed non-proliferating organisms were studied, they were centrifuged at 2000 r.p.m. (average 1000 g) for 2 min at room temperature (22 °C) in a MSE Minor Centrifuge. Resuspension was in 4.5 ml 50 mM citric acid/Na citrate buffer (pH 3.4), followed by rapid recentrifugation in haematocrit tubes and resuspension to a final total

volume of 0.5 ml in the same buffer. Dilution with the same buffer (1.5 ml) was followed by supplementation to a final concentration of 10 mM glucose in the reaction mixture in the O_2 electrode vessel. Some samples were force-aerated with sterile air at 30 °C (200 cm³ min⁻¹) to purge them of volatile fermentation products (e.g. acetaldehyde, H_2S , ethanol, acetic acid): the off-gas was led through a cold-trap kept at 77 K using liquid N_2 .

Measurement of H_2S in the culture off-gas. Exit gas from the oscillating culture was allowed to impinge on a $AgNO_3$ -impregnated test paper; the absorbance of the black deposit of Ag_2S was proportional to the $p(H_2S)$ in the mobile gas phase. Calibration (Vogel, 1954) was with standard solutions of Na_2S brought to pH 3.4 with H_2SO_4 and displacement at 30 °C with an air flow rate of 180 cm³ min⁻¹ (identical to that used for aeration of the continuous culture).

Use of membrane-potential-sensitive fluorophores. For assessment of plasma-membrane electrochemical potential we used 1 µg ml⁻¹ of an oxonol fluorophore, DiBAC₄(3) [bis(1,3-dibutylbarbituric acid)trimethine oxonol] (Dinsdale *et al.*, 1995). Incubation with organisms still suspended in their culture medium was for 30 min at room temperature (22 °C). For the inner mitochondrial membrane potential, Rhodamine 123 (1 µg ml⁻¹) was employed under identical conditions (Chen, 1988). Flow cytometry was performed using a Becton-Dickinson cytometer. Excitation and emission wavelengths were 488 nm and 540 nm, respectively, for both the DiBAC₄(3)- and Rhodamine 123-stained yeasts. For direct fluorimetric estimation of the latter, organisms were harvested at intervals from culture samples incubated with 1 µM Rhodamine 123 using excitation at 540 nm, emission at 580 nm.

Redox state measurements. Samples for cytochrome spectra at high and low DOT (3.8 ml culture) were centrifuged at 1000 g for 2 min in a MSE Minor centrifuge using haematocrit tubes. Resuspension was to a volume of 0.2 ml in 1.0 M mannitol. Reduced-oxidized cytochrome spectra were obtained at 77 K in a Unicam SP 1800 spectrophotometer, using Perspex cuvettes with a light path of 5 or 2 mm. The oxidized sample was aerated and the reduced sample was kept anaerobic immediately before freezing. Samples were thawed and then reequilibrated to 77 K. Spectra were obtained at a band-width of 2.2 nm and with a scan rate of 2 nm s⁻¹ over a spectral range of 400 to 620 nm.

The redox state of the cellular NAD(P)H/NAD⁺ couple was monitored continuously in the culture using the method of Harrison & Chance (1970) using a metabolite fluorimeter (Chance *et al.*, 1975) directly on the fermentation vessel.

Electron microscopy. Samples were fixed as rapidly as possible by rapid sedimentation (Eppendorf Centrifuge, 14000 r.p.m., 30 s) followed by mixing equal volumes with 0.1 M cacodylate buffer pH 6.9 containing 1% paraformaldehyde, 2% glutaraldehyde, 4% (v/v) sucrose and 0.02% $CaCl_2$. After 1 h at 4 °C, fixed organisms were sedimented and then resuspended in 1% OsO_4 at 4 °C for 1 h. Dehydration by passage through a series of ethanol solutions (50, 70 and 90%, 15 min each; 100%, 2 × 30 min at room temp.). Infiltration with Spurr resin was overnight, followed by fresh resin for 8 h and polymerized overnight at 60 °C. Sections of 60–90 nm thickness were obtained using a LKB Ultramicrotome: staining with uranyl acetate was for 15 min, followed by Reynold's lead citrate for 10 min. The microscope was a JEOL 1210 transmission machine.

RESULTS

Oscillatory metabolic changes in the continuous culture

Fig. 1a shows a typical section of the continuously monitored output from the O_2 electrode measuring the DOT in the continuous culture. An autonomous wide-amplitude excursion of the trace from 80 µM O_2 at the respiratory maxima to 140 µM O_2 at the respiratory minima and back occurred every 48 min. Also shown (Fig. 1a) is the NAD(P)H fluorescence emission excited by the 366 nm line of a Hg arc lamp directly illuminating the culture and measured at 450 nm. The redox state of the nicotinamide nucleotide pools oscillated with the same period as that of the respiratory oscillations, and maximum reduction (i.e. maximum fluorescence emis-

Fig. 2. Uncoupling energy conservation in a continuous culture of *S. cerevisiae*. At the solid vertical lines 10 µM CCCP (a, b) or 4 µM 5-13 (c) was injected aseptically.

Fig. 3. Oxygen consumption of samples removed from the oscillating continuous culture of *S. cerevisiae*. (a) Washed non-proliferating organisms suspended in 50 mM sodium citrate buffer (pH 3-4). (b) Culture was diluted 1+3 with the same buffer. At A and B reaeration was for 5 min; distillate was trapped at 77 K and added back at C and D. (c) Non-proliferating organisms incubated in the presence of 10 mM glucose. Examples shown were all from phases of maximal respiration [$6 \text{ nmol O}_2 \text{ min}^{-1} (10^7 \text{ yeast cells})^{-1}$]; similar results were obtained with organisms from phases of minimal respiration [$4 \text{ nmol O}_2 \text{ min}^{-1} (10^7 \text{ yeast cells})^{-1}$]. Numbers shown on the traces indicate rates as a percentage of controls (untreated suspensions). Cell density in the oscillatory culture was 7×10^8 organisms ml^{-1} . Traces shown are typical of results with 5 batches of organisms harvested at respiratory maxima and another 5 samples obtained at respiratory minima.

sion) occurred when DOT was maximal. Fermentation product concentrations also oscillated at this frequency, but with maxima that show distinct phase relationships (Fig. 1a, b). Thus ethanol increased to maxima at around those of DOT, whereas the acetaldehyde (Fig. 1b) phase lagged by about 180° . Fig. 1b shows the time-course of accumulation of H_2S in the off-gas from the

culture during one cycle of respiratory activity. In each cycle, decreasing respiratory activity accompanied the abrupt increase of H_2S .

Uncoupling of mitochondrial energy conservation leads to increased respiration (Hanstein, 1976). Fig. 2 shows the effect of uncoupling respiration from energy con-

Fig. 4. Flow cytometry of *S. cerevisiae* after incubation with 1 $\mu\text{g ml}^{-1}$ membrane-potential-sensitive fluorophores DiBAC₄(3) (a–c), Rhodamine 123 (d–f); (a, c, d, f) organisms from cultures at respiratory minimum; (b, e) organisms from cultures at a respiratory maximum. (g) Uptake of Rhodamine 123 measured directly into organisms from cultures at respiratory minimum (trace 1) and maximum (trace 2). Trace 3, culture sample incubated with 10 μM CCCP.

servation in the continuous culture of *S. cerevisiae*. On addition of 10 μM CCCP (Fig. 2a) or 4 μM S-13 (Fig. 2c), respiration was accelerated; after about 30 min O_2 consumption at both peaks and troughs was increased so that DOT was diminished. During the first cycle after addition of CCCP (Fig. 2b), the period of the oscillation was increased to 60 min; however, after delay times of 11 h and 4 h for CCCP (Fig. 2a) and S-13 (Fig. 2c), respectively, successive cycles showed a rapid restoration to the normal period length (48 min). At the standard dilution rate (1 ml min^{-1}), uncoupler concentration was halved in 13.3 h. Although uncoupling continued (as indicated by accelerated respiration) oscillatory behaviour recovered.

Sensitivities of yeast populations to inhibitors

To determine whether the respiratory oscillation could be attributed to changing electron transport pathways or to alterations of the concentrations of fermentation products during continuous culture, samples of organisms were removed, and washed non-proliferating suspensions assayed for respiratory activities. Fig. 3a shows the rates of respiration of washed yeasts sampled at a peak of DOT and tested at the same pH value as that of the culture (pH 3.4) in Na citrate buffer. Glucose

addition accelerated respiration. To test whether the electron transport pathway alternative to the main phosphorylating respiratory chain was functional, SHAM, an inhibitor specific for the alternative terminal oxidase (Lloyd & Edwards, 1978), was employed. SHAM was not very inhibitory (<10%); subsequent addition of 5 μM NaN₃ gave 90% inhibition and 10 μM resulted in complete inhibition. When the order of use of these two inhibitors was reversed, similar results were obtained, indicating that respiration was mediated almost entirely by the main azide-sensitive electron transport pathway, and this was so for samples taken both at respiratory maxima and minima (not shown). Organisms still in their culture medium transferred to the closed O_2 electrode assay system (Fig. 3b) showed slower O_2 consumption than that of washed organisms. Forced aeration for 2 min (at A and again at B in Fig. 3) gave progressive relief of respiratory inhibition so as to give increased rates of O_2 consumption in both types of sample (i.e. those taken from the culture at respiratory troughs or at peaks showed similar results). This suggests that volatile component(s) that have accumulated in the medium during the culture have inhibited respiration. Addition of the distillate (at C and D in Fig. 3) inhibited respiration. These experiments were repeated at least three times with similar results. Fig. 3c

shows that organisms were not inhibited by 300 mM ethanol. At high concentrations acetaldehyde was inhibitory, but not at those achieved in the cultures. Na_2S was very inhibitory even at micromolar concentrations. The protonophore CCCP accelerated respiration, especially with samples from respiratory minima.

Mixing experiments

To confirm whether accumulation of inhibitor(s) in culture supernatants accounts entirely for changing respiration rate in the continuous culture, cells removed at peak and trough DOT values and then washed, were separately incubated with either type of culture supernatant in the assay system (not shown). Organisms that showed high respiratory rates (harvested at low DOT) continued to utilize O_2 rapidly when incubated with culture supernatant from either peaks or troughs of the oscillatory state. Organisms harvested from respiratory troughs (at high DOT) incubated with either type of medium respired slowly. These mixing experiments indicate that fast or slow respiration rates are primarily determined by states intrinsic to the organisms themselves, although the final rates attained by the culture have minor contributions from fermentation products accumulating in the medium.

Changes in membrane potential

Samples taken at peak and trough DOT values were incubated with $1\ \mu\text{g ml}^{-1}$ of the anionic membrane-potential-sensitive dye DiBAC₄(3). The resulting distribution of fluorescence intensities (Fig. 4a–c) indicated an almost homogeneous population (>98% of the total) showing fluorescence emission similar to that due to autofluorescence (not shown). This indicates that the fluorophore had not entered the organisms. The minor, highly fluorescent subpopulation consists of damaged organisms with greatly diminished integrity of the plasma membrane. Organisms from maximal or minimal respiratory phases of the ultradian cycle show identical properties with respect to exclusion of the oxonol dye (median channels of fluorescence intensities at arbitrary value 11 in all three samples).

Incubation with $1\ \mu\text{g}$ Rhodamine 123 ml^{-1} indicated diminished dye uptake (Fig. 4d, f) during phases of high respiratory rates (low DOT) at median channels 800 in d and 600 in f; the fluorescence emission distribution revealed by flow cytometry is indicative of an enhanced inner mitochondrial membrane potential by comparison with that of organisms sampled at low respiration, high DOT (Fig. 4e, median channel at value 450); direct measurement of fluorophore uptake (Fig. 4g) confirmed this. Cell volume determinations, made using a Coulter Channelyser (not shown), indicated that median values were not measurably different in populations harvested at six successive respiratory maxima and minima. Fig. 4g shows the uptake rates of Rhodamine 123 into the population of organisms at different stages of the respiratory oscillation.

Fig. 5. Low-temperature cytochrome spectra of *S. cerevisiae* at respiratory maxima (a) and minima (b) in a continuous culture. (a, b) Difference spectra: sodium dithionite minus ammonium persulphate oxidized. (c) Direct difference spectra between the continuous culture sampled (at low and high DOT; respiratory maximum minus respiratory minimum).

Cytochromes

Difference spectra (sodium dithionite reduced minus ammonium persulphate oxidized) indicate that cytochromes *c*, *c*₁, *b* and *a*+*a*₃ are not altered in total amounts between the samples taken at respiratory maxima (Fig. 5a) and minima (Fig. 5b). However, when culture samples were rapidly quenched to 77 K and difference spectra were obtained directly, the resulting absorption maxima (Fig. 5c) indicated that the steady-state redox balances were different between the maxima and minima of respiration. In the samples from cultures at low DOT, cytochromes *c* and *a*+*a*₃ were more oxidized than in those at high DOT.

Electron microscopy

At low respiration rates (Fig. 6a) mitochondrial section areas appear large and the cristae were more distinct than those of organisms fixed during the phases of high respiration (Fig. 6b). Other changes also occur; the cell wall structure appeared to be altered in the stage of low respiration.

Fig. 6. Electron micrographs of thin sections of organisms sampled at low DOT (high respiration) (a–c) or high DOT (low respiration) (d–f) states of the continuous culture, respectively. Bars, 2 μm (a, d); 0.5 μm (b, e); 0.2 μm (c, f).

DISCUSSION

Energy conservation has been studied extensively using 'intact' mitochondria prepared after gentle isolation procedures applied initially to mammalian tissues; subsequently functional mitochondria were isolated from *S. cerevisiae* (Ohnishi *et al.*, 1966). Irrespective of their source, the respiratory rate of mitochondria *in vitro* is determined by the presence of a respiratory substrate (e.g. NADH, succinate or malate), O_2 and the requirement for oxidative phosphorylation (ADP and P_i , Fig. 7). The respiratory states that correspond to conditions where these various requirements are satisfied were defined in a series of publications (reviewed by Chance & Williams, 1956). Thus the fully active respiratory system of isolated mitochondria (designated as 'energized' or #3) may under favourable conditions show a rate of O_2 consumption as much as 10-fold

greater than those in the resting or 'de-energized' (#4) state. When a respiratory substrate, O_2 and P_i are supplied, control of respiration can be demonstrated on making small additions of ADP, as reversible transitions between respiratory states #3 and #4 (i.e. increased and decreased O_2 consumption rates as measured using a closed O_2 electrode system); after the completion of phosphorylation of the added pulse of ADP, the respiratory activity returns to its initial resting (#4) rate. The effect of protonophore-mediated uncoupling of energy conservation is to increase the respiration of resting mitochondria by short-circuiting the proton translocating ATP synthase and thereby abolishing the dependence of electron transport rates on transmembrane electrochemical potential (Mitchell & Moyle, 1969). This results in a rapidly respiring uncoupled (state 3u) respiratory condition, in which the membrane potential is collapsed.

Fig. 7. Changes in mitochondrial structure that accompany altered metabolic status during energy conservation.

These physiological states of mitochondria correspond to distinctive morphological conditions, and the metabolic transitions described above are accompanied by characteristic ultrastructural changes that occur not only *in vitro* but also *in situ* (Hackenbrock, 1968a, b). Thus, in resting mitochondria, the cristae are clearly visible, folded into sheets, and most of the internal space is occupied by the matrix. On activation of respiration and oxidative phosphorylation, the intermembrane space expands at the expense of a decrease in the matrix. These conformations are referred to as 'orthodox' and 'condensed' respectively (Fig. 7).

The results presented here strongly suggest that the ultradian-clock-driven oscillatory response of mitochondrial respiration involves continuous cycles of energization and de-energization. There was at any time little detectable flow of electrons through a SHAM-sensitive alternative terminal oxidase and thus electron apportionment did not detectably change at any stage of the oscillation. Experiments in which organisms and medium from the two extremes of respiratory activity were mixed confirm that the observed changes are intrinsic to the organisms and do not primarily arise from alteration of medium composition.

Thus during the stage of high DOT (low respiratory activity), mitochondrial transmembrane electrochemical potential is increased, and in section in electron micrographs the mitochondria appear to be in the 'orthodox' conformation (Hackenbrock, 1968a, b). In this state, the organelles are characterized by a relatively large matrix volume and with the inner boundary membrane (the non-cristae component of the inner mitochondrial membrane) closely apposed to the outer membrane, with only a small space between them. In this stage, mitochondrial cytochrome *b* was more highly

reduced, as were the intracellular pools of NAD(P)H and the total flavoprotein.

In the phases of low DOT (high respiratory activity) the mitochondria assume the 'condensed' conformation, in which the inner membrane is pulled away from the outer membrane and the cristae are less clearly defined, the total nicotinamide nucleotide pool is more oxidized as are the cytochromes *c* and *a + a₃*. Thus the succession of events during respiratory oscillation in yeast is similar to that previously described in the soil amoeba *Acanthamoeba castellanii* (Edwards & Lloyd, 1978; Lloyd *et al.*, 1982a, b) where it was concluded that the phenomenon represents *in vivo* mitochondrial respiratory control (ADP-acceptor control; Chance & Williams, 1956). The slow kinetics indicates that, as in the amoeba, the cycle of mitochondrial changes in yeast is responding to a slowly changing energetic demand of biosynthetic processes (Edwards & Lloyd, 1980; Lloyd *et al.*, 1981), i.e. that it is controlled by an ultradian clock (Lloyd & Edwards, 1984, 1987). That the respiratory oscillation in *S. cerevisiae* has a temperature-compensated period and therefore has a timekeeping function has recently been shown (Murray *et al.*, 2001). The relaxation time of the oscillating control circuit that determines the 48 min cycle time lies in a slower time domain than that characteristic of mitochondrial energy metabolism (milliseconds to seconds); it belongs rather in the domain that typically includes the processes of transcription and translation.

Other changes (e.g. the oscillatory accumulation of the fermentation products ethanol, acetaldehyde and H_2S) may contribute to the respiratory oscillation. During glucose-supported growth, the ethanol concentration varied from 110 to 125 mM, whereas acetaldehyde ranged from 0.4 to 0.85 mM, and H_2S varied between

1.5 μ M and an undetectable concentration. Thus the respiratory inhibitor H_2S reaches its highest concentration in the culture when respiration is at its lowest. However, H_2S never exceeded 1.5 μ M, and at this concentration, inhibition of yeast respiration was less than 10%. Inhibition by acetaldehyde must also occur, but not at the low concentrations (<0.75 mM) monitored during continuous culture (50% inhibition requires 100 mM and its maximum accumulation corresponds to maximum respiration).

Unlike the oscillatory respiratory metabolism studied in *A. castellanii* (Edwards & Lloyd, 1978, 1980; Lloyd *et al.*, 1982a) or *Schizosaccharomyces pombe* (Poole *et al.*, 1973), that of *Sacch. cerevisiae* reported here is not directly coupled to the cell division cycle, i.e. does not require cultures in which the growth and division of the population is synchronized for its detection. Yet there must be synchronization of individual organisms to generate coherence in the populations so as to result in an observable oscillatory behaviour. Rather, in this case there is *metabolic* coupling between individual cells of the culture. It has been proposed that highly diffusible volatile low molecular weight compound(s), e.g. acetaldehyde and H_2S , may act as intercellular signalling substances required to maintain coherence of the population (Keulers & Kuriyama, 1998; Sohn *et al.*, 2000; Sohn & Kuriyama, 2001). This must be a rapid process (with a time scale of seconds rather than minutes) to coordinate the oscillatory behaviour with such precision (Klevecz & Murray, 2001).

Further work is required to determine how the oscillation is controlled, i.e. the extramitochondrial events that produce the slow kinetics of transcriptional switching that generate the time-base of the temperature-compensated ultradian clock (Murray *et al.*, 2001; Salgado *et al.*, 2002). The continuously monitored system described here for study of ultradian clock outputs in *S. cerevisiae* is more convenient and amenable than other ultradian systems (Lloyd, 1992) and many circadian systems (Lloyd *et al.*, 1982b; Lloyd, 1988) where only discrete sampling is possible (Lloyd & Murray, 2000), and where the accumulation of useful data requires more extended time intervals.

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REFERENCES

- Chance, B. & Williams, R. G. (1956). The respiratory chain and oxidative phosphorylation. *Adv Enzymol Relat Areas Mol Biol* **17**, 65–134.
- Chance, B., Legallais, V., Sorge, J. & Graham, N. (1975). A versatile time-sharing, multichannel spectrophotometer, reflectometer and fluorometer. *Anal Biochem* **66**, 498–514.
- Chen, L. B. (1988). Mitochondrial membrane potential in living cells. *Annu Rev Cell Biol* **4**, 155–181.
- Dinsdale, M. G., Lloyd, D. & Jarvis, B. (1995). Yeast vitality during cider fermentation: two approaches to the measurement of membrane potential. *J Inst Brew* **101**, 453–458.
- Edwards, S. W. & Lloyd, D. (1978). Oscillations of respiration and adenine nucleotides in synchronous cultures of *Acanthamoeba castellanii*. *J Gen Microbiol* **108**, 197–204.
- Edwards, S. W. & Lloyd, D. (1980). Oscillations in protein and RNA content during synchronous growth of *Acanthamoeba castellanii*. *FEBS Lett* **109**, 21–26.
- Hackenbrock, C. R. (1968a). Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states. *Proc Natl Acad Sci USA* **61**, 598–605.
- Hackenbrock, C. R. (1968b). Ultrastructural bases for metabolically-linked mechanical activities in mitochondria. II. Electron transport-linked ultrastructural transformation in mitochondria. *J Cell Biol* **37**, 345–369.
- Hanstein, W. G. (1976). Uncoupling of oxidative phosphorylation. *Biochim Biophys Acta* **456**, 129–148.
- Harrison, D. E. F. & Chance, B. (1970). Fluorimetric technique for monitoring changes in the level of reduced nicotinamide nucleotides in continuous cultures of microorganisms. *Appl Microbiol* **19**, 446–450.
- Keulers, M. & Kuriyama, H. (1998). Extracellular signalling in oscillatory yeast culture. In *Information Processing Cells and Tissues*, pp. 85–94. Edited by W. M. L. Holcombe, R. Paton and M. Holcombe. New York: Plenum.
- Keulers, M., Suzuki, T., Satroutdinov, A. D. & Kuriyama, H. (1996a). Autonomous metabolic oscillation in continuous culture of *Saccharomyces cerevisiae* growth on ethanol. *FEMS Microbiol Lett* **142**, 253–258.
- Keulers, M., Satroutdinov, A. D., Suzuki, T. & Kuriyama, H. (1996b). Synchronisation affecter of autonomous short-period-sustained oscillation of *Saccharomyces cerevisiae*. *Yeast* **12**, 673–682.
- Klevecz, R. R. & Murray, D. B. (2001). Genome wide oscillations in expression. *Mol Biol Rep* **28**, 73–82.
- Lloyd, D. (1974). *The Mitochondria of Microorganisms*. London: Academic Press.
- Lloyd, D. (1988). Circadian and ultradian clock-controlled rhythms in unicellular microorganisms. *Adv Microb Physiol Biochem* **39**, 291–298.
- Lloyd, D. (1992). Intracellular timekeeping: epigenetic oscillations reveal the functions of an ultradian clock. In *Ultradian Rhythms in Life Processes*, pp. 5–22. Edited by D. Lloyd and E. R. Rossi. London: Springer.
- Lloyd, D. & Edwards, S. W. (1978). Electron transport pathways alternative to the main phosphorylating chain. In *Functions of Alternative Oxidases*, pp. 1–10. Edited by H. Degn, D. Lloyd and G. C. Hill. Oxford: Pergamon.
- Lloyd, D. & Edwards, S. W. (1984). Epigenetic oscillations during the cell cycles of lower eukaryotes are coupled to a clock: life's slow dance to the music of time. In *Cell Cycle Clocks*, pp. 27–46. Edited by L. N. Edmunds. New York: Marcel Dekker.
- Lloyd, D. & Edwards, S. W. (1987). Temperature-compensated ultradian rhythms in lower eukaryotes: timers for cell cycle and circadian events? In *Advances in Chronobiology, Part A*, pp. 131–151. Edited by J. E. Pauly and L. E. Scheving. New York: Alan R. Liss.
- Lloyd, D. & Murray, D. S. (2000). Redox cycling of intracellular thiols: state variables for ultradian, cell division cycle and circadian cycles. In *Redox Behaviour of Circadian Systems*, pp. 85–94. Edited by T. Vanden Driessche, J.-L. Guisnet and G. P. De Vries. Amsterdam: Kluwer.

- Lloyd, D., Edwards, S. W. & Williams, J. L. (1981).** Oscillatory accumulation of total cellular protein in synchronous cultures of *Candida utilis*. *FEMS Microbiol Lett* **12**, 295–298.
- Lloyd, D., Edwards, S. W. & Fry, J. C. (1982a).** Temperature-compensated oscillations in respiration and cellular protein content in synchronous cultures of *Acanthamoeba castellanii*. *Proc Natl Acad Sci USA* **79**, 3785–3788.
- Lloyd, D., Poole, R. K. & Edwards, S. W. (1982b).** *The Cell Division Cycle: Temporal Control of Cellular Growth and Reproduction*. New York: Academic Press.
- Luzikov, V. N. (1984).** Mechanisms of mitochondrial stabilization. In *Mitochondrial Biogenesis and Breakdown*, pp. 109–114. New York: Plenum.
- Mitchell, P. & Moyle, J. (1969).** Estimation of membrane potential and pH difference across the cristal membrane of rat-liver mitochondria. *Eur J Biochem* **7**, 471–478.
- Murray, D. B., Engelen, F. A., Keulers, M., Kuriyama, H. & Lloyd, D. (1998a).** NO⁺ but not NO[•] inhibits respiratory oscillations in ethanol-grown chemostat cultures of *Saccharomyces cerevisiae*. *FEBS Lett* **431**, 297–299.
- Murray, D. B., Engelen, F. A., Keulers, M., Kuriyama, H. & Lloyd, D. (1998b).** NO⁺ but not NO[•] inhibits respiratory oscillations in ethanol-grown chemostat cultures of *Saccharomyces cerevisiae*. *Trans Biochem Soc* **26**, S339.
- Murray, D. B., Engelen, F., Lloyd, D. & Kuriyama, H. (1999).** Involvement of glutathione in the regulation of respiratory oscillation during a continuous culture of *Saccharomyces cerevisiae*. *Microbiology* **145**, 2739–2745.
- Murray, D. B., Roller, S., Kuriyama, H. & Lloyd, D. (2001).** Clock control of ultradian respiratory oscillation found during yeast continuous culture. *J Bacteriol* **183**, 7253–7259.
- Ohnishi, T., Kawaguchi, K. & Hagihara, B. (1966).** Isolation of intact mitochondria from yeast. *J Biol Chem* **241**, 1797–1806.
- Poole, R. K., Lloyd, D. & Kemp, R. B. (1973).** Respiratory oscillations and heat evolution in synchronously dividing cultures of the fission yeast *Schizosaccharomyces pombe* 972h[–]. *J Gen Microbiol* **77**, 209–220.
- Salgado, L. E. J., Murray, D. B. & Lloyd, D. (2002).** Some anti-depressant agents (Li⁺, monoamine oxidase type A inhibitors) perturb the ultradian clock in *Saccharomyces cerevisiae*. *Biol Rhythm Res* **33**, 351–361.
- Satroudinov, A. D., Kuriyama, H. & Kobayashi, H. (1992).** Oscillatory metabolism of *Saccharomyces cerevisiae* in continuous culture. *FEMS Microbiol Lett* **98**, 261–268.
- Sohn, H.-Y. & Kuriyama, H. (2001).** Ultradian metabolic oscillation of *Saccharomyces cerevisiae* during aerobic continuous culture: H₂S, a population synchroniser is produced by sulphite reductase. *Yeast* **18**, 125–135.
- Sohn, H.-Y., Murray, D. B. & Kuriyama, H. (2000).** Ultradian oscillation of *Saccharomyces cerevisiae* during aerobic continuous culture: hydrogen sulphide mediates population synchrony. *Yeast* **16**, 1185–1190.
- Vogel, A. I. (1954).** Reactions of sulphides. In *Macro and Semimicro Qualitative Inorganic Analysis*, 4th edn, p. 336. London: Longmans, Green & Co.

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