The Influence of Growth Substrate and Capacity for Oxidative Phosphorylation on Respiratory Oscillations in Synchronous Cultures of *Escherichia coli* K12

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SUMMARY

Fluctuations in cell volume during exponential growth of *Escherichia coli* K12 changed the effectiveness of the continuous-flow centrifugation method for preparing synchronous cultures. Rates of oxygen uptake in synchronous cultures were measured using an electrode system open to the atmosphere. In synchronous cultures of both the parental strain and an adenosine triphosphatase-deficient mutant, which was incapable of oxidative phosphorylation, respiration rates doubled during the cell cycle but oscillated with a periodicity of approximately half a cycle. Synchronous cultures of the parental strain growing on glycerol and Casamino acids showed a stepwise pattern of oxygen consumption. Continuous flow centrifugation did not markedly affect the increases in the numbers and respiration rates of cells in synchronous cultures. Respiratory oscillations also occurred on inoculation of a late-stationary phase culture into fresh medium, although synchronous division was not observed. The possible mechanisms underlying respiratory fluctuations under different growth conditions are discussed.

INTRODUCTION

Rates of respiration during the cell cycles of a variety of eukaryotic micro-organisms have been extensively studied (see Lloyd, 1974) but the biochemical and physiological bases for the complex patterns that are often observed are poorly understood. During synchronous growth in a defined medium containing alanine as carbon source after selection by continuous-flow centrifugation, respiration rates of *Escherichia coli* oscillated with a periodicity of about one-third of a cell cycle (Evans, 1975). In this paper the physiological basis of these oscillations has been studied by examining the effects of (i) growth with alternative carbon sources and (ii) a genetic lesion in the membrane-bound adenosine triphosphatase (ATPase). It is also shown that respiratory oscillations may persist independently of synchronous cell growth and division. Parts of this work have been briefly communicated previously (Poole, 1975).

METHODS

Organisms and growth conditions. *Escherichia coli* strains A1002 and A103c were kindly supplied by Dr B. A. Haddock. Strain A1002 (K12 Y *mel ilv⁻ lacI⁻ metE⁻*) is the parental strain from which the ATPase-deficient mutant, A103c, was derived (Schairer & Haddock, 1972). Both were grown at 37 °C in mineral salts medium (Poole, 1977) containing either 0.5 % (w/v) glucose or 0.5 % (w/v) glycerol as carbon source, in the absence or presence of 0.1 % (w/v) vitamin-free Casamino acids (Difco). Strain A103c was regularly screened for

reversion by checking its inability to grow on solid defined medium containing only acetate, malate and succinate (all at 0.2 %, w/v) as carbon sources (Schairer & Haddock, 1972). Glucose-containing cultures (2 l) were grown under forced aeration and with magnetic stirring. Glycerol-containing cultures were grown with more vigorous aeration by shaking 200 ml volumes in 1 l conical flasks at 200 rev. min⁻¹. Synchronous cultures (200 ml) were grown, with magnetic stirring, in an open vessel fitted with a heating coil. Alternatively, 2 ml portions of concentrated culture were grown in the open reaction vessel of an oxygen electrode, as described below.

Preparation of synchronous cultures by continuous-flow centrifugation. This was as described previously (Poole, 1977). The total centrifugal force (g -min) was calculated for a radius of 2.9 cm (half-maximal radius) using the mean residence time of the culture in the rotor (rotor volume, ml/flow rate, ml min⁻¹). The resulting synchronous culture was used either directly or after concentration by rapid filtration under reduced pressure through a 50 mm diam. Nufflow membrane filter (0.45 μ m pore size; Oxoid). Cells were recovered quantitatively on the filter and immediately resuspended in a small volume of the filtrate. Zero time for the synchronous culture was taken as the time when half the effluent volume subsequently used for analysis had been collected.

Cell numbers and volumes. These were determined using a Coulter counter model Z₈₁ and Channelyzer C1000 (Coulter Electronics, Harpenden, Hertfordshire) as described by Poole (1977).

Assessment of synchrony. Synchrony indices calculated for the first (F_1) and second (F_2) increases in cell numbers (midpoints indicated by vertical lines in the figures) are those of Blumenthal & Zahler (1962). For an exponentially growing culture, the value of the index is 0; for a theoretical culture exhibiting perfect synchrony, its value is 1.0.

Measurements of oxygen uptake. Oxygen uptake rates in 2 ml culture samples were measured using an O₂ electrode (Rank Bros, Bottisham, Cambridge) calibrated as described by Chappell (1964). Respiratory inhibitors and uncoupling agents were added as small volumes of concentrated aqueous or methanolic solutions; control experiments showed that these concentrations of methanol alone had no effect on respiration rates. Possible errors in the determination of respiratory patterns in rapidly growing cultures, arising from infrequent sampling, were overcome by obtaining a continuous recording of O₂ tension in small cultures in an O₂ electrode system open to the atmosphere, similar to that described by Degn, Lilleør & Iversen (1973). The standard reaction vessel (Rank Bros) was a cylindrical well, 1.6 cm diam. $\times$ 4.0 cm depth, at the bottom of which was a Clark-type O₂ electrode and a magnetic stirrer bar. The cap that normally seals the contents of the vessel from the atmosphere was replaced with a bung fitted with inlet and outlet tubes through which a slow stream of water-saturated air was passed to minimize evaporation from the sample. The maximum rate of evaporation for a vigorously stirred 2 ml culture sample was about 3×10^{-3} ml min⁻¹ at 35 °C. The O₂ transfer constant, K , was determined from the observed half-time of the first-order equilibration of liquid (made anoxic with a grain of dithionite) with atmospheric O₂ (Degn *et al.*, 1973). K was measured in each experiment; the values were in the range 0.41 to 0.51 min⁻¹.

When temperature, sample volume and rate of stirring are constant, the measured variations in dissolved O₂ tension reflect the changing respiration rates of cells in the culture. In a control experiment, 2 ml of air-saturated growth medium without cells was incubated for 150 min under the conditions described above; fluctuations in the recorded O₂ tension were less than 2 % of the total over this period. Samples (10 μ l) of culture were removed from a second identical vessel and diluted in electrolyte for determinations of cell numbers.

Fig. 1

Fig. 1. Mean cell volume as a function of cell population during asynchronous growth of *E. coli* strains A1002 and A103c in media containing glucose or glycerol. Cell numbers and volumes were determined in formaldehyde-fixed (final concn 0.4 %) culture samples. ○, Strain A103c grown with glucose and Casamino acids, doubling time 48 min; ●, strain A1002 grown with glucose and Casamino acids, doubling time 44 min; ■, strain A1002 grown with glycerol and Casamino acids, doubling time 40 min; □, strain A1002 grown with glycerol, no Casamino acids, doubling time 126 min. Arrows indicate the limits of the periods during which the cultures were routinely used for continuous-flow centrifugation.

Fig. 2

Fig. 2. Oxygen uptake in a synchronous culture of *E. coli* strain A1002 grown with glucose and Casamino acids. The population at the time of selection was 4.4×10^8 cells ml^{-1} . Flow rate of the culture through the continuous action rotor was 161 ml min^{-1} and the rotor speed $1590 \text{ rev. min}^{-1}$ ($2.2 \times 10^4 \text{ g-min}$ at half-maximal radius). After discarding the first 300 ml of effluent, a portion (75 ml), which contained 6.9 % of the original culture, was collected and concentrated 13-fold. Samples (2 ml) were cultured in open O_2 electrode vessels. ●, Cell numbers; ○, O_2 uptake rates calculated at intervals corresponding to 2.5 min from the polarographic trace and expressed as $\text{ng-atom O min}^{-1} \text{ ml}^{-1}$. F_1 and F_2 denote the synchrony indices of the first and second doublings of the cell numbers, respectively.

Table 1. *Effect of growth phase on the proportion of glucose-grown E. coli strain A1002 escaping retention in the continuous action rotor*

Cell population when centrifuged ($10^{-8} \times \text{organisms ml}^{-1}$)	$10^{-4} \times \text{total centrifugal}$ force (g-min)	Proportion of original culture recovered in rotor effluent (%)
2.1	2.27	13.2
4.4	2.20	6.9
7.3	2.30	3.5

Extinction. The extinction of culture samples was measured in cuvettes of 1 cm light path using a Pye Unicam SP1700 spectrophotometer.

Chemicals. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was from Sigma; dibromothymoquinone was a generous gift from Dr B. A. Haddock. All other reagents were of the highest purity commercially available.

RESULTS

Fluctuations in cell volume during asynchronous growth

During asynchronous growth of an unfractionated culture the mean cell volume fluctuated markedly (Fig. 1). Cells of strains A1002 and A103c, grown with glucose and Casamino acids, decreased in volume until the population reached about 2×10^8 organisms ml^{-1} and then, fluctuating by less than 6 % for three doublings, their volume increased slowly as the

Fig. 3. Cell cycle maps of timings of peaks in O_2 uptake during the cell cycle of *E. coli* grown with glucose and Casamino acids. In (a) and (b) the abscissae represent (as a linear scale) the cell cycle taken as the period between the midpoints of the first and second increases of cell numbers in synchronous cultures. The triangles ($\blacktriangledown$) denote the timing of the peaks of O_2 uptake during the cell cycle of (a) strain A1002 and (b) strain A103c. Arrows with crossbars indicate mean values with standard deviations.

population approached 1×10^8 organisms ml^{-1} . Cells grown with glycerol and Casamino acids continued to decrease in mean volume (about $0.07 \mu m^3$ per doubling time) over the period examined; in the absence of Casamino acids, the fluctuation in cell volume was less marked. These results confirm and extend previous reports (Stephenson, 1949; Zaritsky & Pritchard, 1973; Smither, 1975).

The effect of these fluctuations, and consequently of the rate of sedimentation, on the recovery of glucose-grown strain A1002 in the rotor effluent is shown in Table 1. Under otherwise similar conditions, the proportion of cells in the rotor effluent depended on the phase of growth, being inversely proportional to the cell population at the time of centrifuging. Strict standardization of the age and size of the inoculum and of the phase of growth at harvest (limited by the arrows in Fig. 1) eliminated wide variations in the proportion of the original culture recovered.

Oxygen uptake in synchronous cultures of strain A1002 grown with glucose and Casamino acids

When the effluent culture from the continuous action rotor was concentrated 13-fold and incubated in an open electrode vessel, the numbers of cells increased by 30 % during the first 20 min of the experiment; thereafter two synchronous doublings of numbers occurred, with good synchrony indices (Fig. 2a). The first complete cell cycle (indicated by the vertical bars) took 42 min. In seven similar experiments the mean value was 44 min (S.D. ± 5.4), equal to the mean generation time of exponentially growing cultures.

The respiration rate, calculated from the continuous recorder trace of O_2 concentration (Degn *et al.*, 1973), doubled in a period equal to the length of a cycle, but rose to two maxima in each cell cycle, one just before the midpoint of cell division and the other in the first half of the cycle (Fig. 2b). Oscillations of the same periodicity were consistently observed in seven similar experiments. The mean positions of the peaks measured in 10 cell cycles were 0.45 and 0.95 of the cycle (Fig. 3a). The amplitude of the oscillations (i.e. peak to trough) was more variable, ranging from 7 to 17 % of the total respiration rate measured at the peaks.

Rates of O_2 uptake were also measured (using a conventional oxygen electrode) in samples withdrawn at intervals from a larger synchronous culture that had not been concentrated by filtration (results not shown). Two experiments confirmed that the rates of respiration oscillated and doubled during the cell cycle. Using this method of analysis the respiratory discontinuities could not be fully resolved due to the relative infrequency with which samples could be removed and analysed. The amplitude of the oscillations was always greater at the beginning of the experiments and subsequently decreased as the degree of synchrony of growth decayed.

A control experiment, described below, showed that growth and respiration of cultures were not affected by centrifugation, the short exposure to sub-optimal temperatures or rapid filtration. A culture (2.7×10^8 cells ml^{-1}) was passed through the continuous action rotor, the centrifuge was stopped, and the thick cell suspension within the rotor was mixed with the effluent, which contained 5 % of the initial population, and vigorously aerated at 37 °C. Exponential increases in the number of cells, the extinction of the culture and the O_2 uptake rate (measured using a conventional O_2 electrode system) were all similar to those before selection and showed no discontinuities. When such a culture was concentrated by filtration and incubated in the open electrode, a similar exponential increase in O_2 uptake rate was observed.

Oxygen uptake in a synchronous culture of the ATPase-deficient mutant

Strain A103c lacks the reversible membrane-bound Mg^{2+} -dependent ATPase and so cannot utilize a transmembrane proton motive force for ATP synthesis (Schairer & Haddock, 1972). After continuous-flow selection, a lag of about 30 min preceded the onset of the first increase in the number of cells: the length of the subsequent complete cell cycle was 70 min (Fig. 4*a*). Though the rate of O_2 uptake increased exponentially overall during the experiment, doubling during a cell cycle, it still oscillated (Fig. 4*b*). The first peak of uptake occurred before the onset of division in the population and the second coincided with the midpoint of division. This pattern was repeated in the next cycle; similar results were obtained in a second experiment. The observed patterns of respiration during the cell cycle of the mutant (Fig. 3*b*) are thus similar to those of its parental strain.

Oxygen uptake in synchronous cultures of strain A1002 grown with glycerol and Casamino acids

To investigate the contribution of catabolite repression by glucose to the observed oscillations, glycerol was used as an alternative carbon source. Respiration rates per cell in glycerol-containing cultures were two- to threefold higher than those of glucose-containing cultures. The length of the first complete cycle was identical to the mean generation time of exponential cultures (40 min). Synchronous growth was maintained for at least two complete cycles (Fig. 5*a*). In contrast to the oscillating rates of respiration observed in glucose-containing cultures (Fig. 2*b*), stepped patterns of respiration were obtained (Fig. 5*b*). In measuring the periodicity of the steps, the inflections between the plateaus and the rapid rises (steps) in respiration rates were used as easily identifiable points. Using this convention the mean periodicity of the steps observed in two separate experiments (one of which is shown in Fig. 5) was 0.76 of a cell cycle (S.D. ± 0.11). This phenomenon has been observed previously by Scopes & Williamson (1964) in synchronous cultures of *Saccharomyces cerevisiae* prepared by starvation. They suggested that the timing 'clock' for growth (reflected in respiration rates) may be dissociated from the control of cell division.

Sensitivity of intact cells to respiratory inhibitors and uncoupling agents

Titration of the varying respiration rates in synchronous cultures of eukaryotic micro-organisms with inhibitors of electron transport or uncouplers of energy conservation has helped in understanding the mechanisms underlying the respiratory discontinuities (e.g. Poole, Lloyd & Kemp, 1973). Similar experiments with *E. coli* were frustrated by the insensitivity or unreproducibility of the response of intact cells to a range of respiratory poisons. Although KCN (at 1.5 mM) gave greater than 90 % inhibition of O_2 uptake by strain A1002 grown with glycerol, this was not achieved until 3 to 4 min after adding the

Fig. 4

Fig. 5

Fig. 4. Oxygen uptake in a synchronous culture of *E. coli* strain A103C grown with glucose and Casamino acids. The population at the time of selection was 3.3×10^8 cells ml^{-1} . Flow rate of the culture through the continuous action rotor was 164 ml min^{-1} and the rotor speed $14\,700 \text{ rev. min}^{-1}$ ($1.85 \times 10^4 \text{ g-min}$ at half-maximal radius). After discarding the first 300 ml of effluent, a portion (50 ml), which contained 10% of the original culture, was collected and concentrated 10-fold. Samples (2 ml) were cultured in open O_2 electrode vessels. ●, Cell numbers; ○, O_2 uptake rates calculated at intervals corresponding to 2.5 min from the polarographic trace and expressed as $\text{ng-atom O min}^{-1} \text{ ml}^{-1}$. F_1 and F_2 denote the synchrony indices of the first and second doublings of the cell numbers, respectively.

Fig. 5. Oxygen uptake in a synchronous culture of *E. coli* strain A1002 grown with glycerol and Casamino acids. The population at the time of selection was 3.3×10^8 cells ml^{-1} . Flow rate of the culture through the continuous action rotor was 170 ml min^{-1} and the rotor speed $15\,000 \text{ rev. min}^{-1}$ ($1.85 \times 10^4 \text{ g-min}$ at half-maximal radius). After discarding the first 300 ml of effluent, a portion (200 ml), which contained 8.6% of the initial population, was collected and concentrated 10-fold. Samples (2 ml) were cultured in open O_2 electrode vessels. ●, Cell numbers; ○, O_2 uptake rates calculated at intervals corresponding to 2.4 min from the polarographic trace and expressed as $\text{ng-atom O min}^{-1} \text{ ml}^{-1}$. F_1 and F_2 denote the synchrony indices of the first and second doublings of the cell numbers, respectively.

inhibitor. Dibromothymoquinone (at 0.5 mM) had no effect on O_2 uptake by intact cells up to 2 min after its addition; yet at 0.1 mM it gives 20% inhibition of oxidase activity of membrane particles and at 0.5 mM totally inhibits growth (Poole & Haddock, 1975). Occasionally, stimulation of respiration rates (20 to 50%) was achieved by dibromophenol, 2,4-dinitrophenol or pentachlorophenol (all at $10 \mu\text{M}$) or by CCCP ($2 \mu\text{M}$). These effects were not reproducible and were of limited value in quantitative measurements of the sensitivity of cells at various stages of the cell cycle.

Respiratory oscillations in the absence of synchronous growth

Oscillations in the rate of O_2 uptake were also observed after perturbation of growth conditions in the absence of synchronous growth. Cells from a 16 h late-stationary phase culture were diluted in fresh medium containing glucose and Casamino acids. The number of cells increased gradually after 30 min incubation until the normal exponential growth rate was achieved after about 90 min (Fig. 6a). Although the increase in the extinction of the culture was discontinuous (Fig. 6b), there was no evidence of synchronous cell division. The induction of synchrony by transfer of stationary phase cells to fresh medium is critically

Fig. 6. Changes in cell population and respiration rate on inoculating a stationary phase culture of *E. coli* A1002 into fresh glucose-containing medium. Cell numbers (●) and extinction (○) were determined in formaldehyde-fixed samples removed from a culture which had been inoculated at a 1:400 dilution with cells from a stationary phase culture (2×10^8 cells ml^{-1}). For determination of O_2 uptake rates (■, □; duplicate expts), the stationary phase culture was diluted 40-fold and incubated in an open O_2 electrode vessel: uptake rates are expressed as $\text{ng-atom O min}^{-1} \text{ ml}^{-1}$.

dependent on the period within the stationary phase when cells are taken (Cutler & Evans, 1966). Oxygen uptake, measured in an open electrode system in a parallel experiment, oscillated markedly during the first 60 min of growth, with a periodicity of about 20 min (Fig. 6c). These oscillations were reproduced in a second experiment (Fig. 6d).

DISCUSSION

Despite its many advantages (Lloyd *et al.*, 1975; Evans, 1975), continuous-flow age selection may result in extended cell cycle times (Fig. 4). Sometimes, the period between collection of the rotor effluent and the first synchronous division is considerably less than the length of subsequent cycles (Figs 4 and 5), or the first division is completed immediately after establishing the synchronous culture (Fig. 2). This is probably a result of the fluctuations in the buoyant density of cells that occur during the cell cycle of this organism (Poole, 1977). Furthermore, when the technique is applied to cultures grown with glycerol in the absence of Casamino acids, cells collected in the rotor effluent divided asynchronously with a doubling time considerably greater than that of the unfractionated culture (unpublished observations).

In constructing cell cycle maps (Fig. 3), the short periods preceding the first division have been regarded as unperturbed latter portions of the normal cycle, since control experiments showed that the continuous-flow procedure caused little disturbance of growth and respiration.

The measurement of respiration rates of cultures using a continuous recording of the O_2 tension has obvious advantages where conventional methods of sampling and subsequent analysis offer insufficient time resolution. The continuous nature of these measurements makes repeated determinations on identical samples and the assessment of experimental

precision difficult. However, the observed respiratory fluctuations were reproducible in replicate experiments and were much greater than the small fluctuations observed in the absence of respiring cells caused by changes in sample volume, temperature and stirring rate. The principal disadvantages of the method are: (i) the rate of stirring and thus of aeration must be chosen to maintain O_2 -sufficient growth conditions throughout the experiment, whilst allowing the changing respiration of cells to cause measurable changes in dissolved O_2 tension; (ii) in order to avoid marked changes in culture volume, the number of cells must be determined in samples from a second vessel; and (iii) the effect of respiratory inhibitors on O_2 uptake rates at various stages of growth cannot be studied without also inhibiting growth.

In this study two peaks of O_2 uptake, rather than three (Evans, 1975), were observed during each cell cycle and these were replaced by a stepped pattern of uptake in cultures grown with glycerol, a non-catabolite-repressive substrate. These observations resemble those obtained with the highly-fermentative fission yeast *Schizosaccharomyces pombe* in three respects. (i) During the cell cycle of cells grown with glucose, the respiratory activity oscillates with a periodicity of half a cycle (Poole *et al.*, 1973). (ii) Growth in the presence of a non-catabolite-repressive carbon source, glycerol, results in stepwise increases in O_2 uptake (Poole & Lloyd, 1974). (iii) Oscillations observed with glucose as carbon source do not appear to be due to respiratory control *in vivo*, i.e. the rate limitation of O_2 consumption by the availability of ADP in the presence of P_i (Chance & Williams, 1956). This conclusion is supported, in the case of *E. coli*, by the persistence of the oscillations in a mutant incapable of oxidative phosphorylation and, in *S. pombe*, by the finding that CCCP preferentially stimulates rates of O_2 uptake and heat evolution when respiration rates are at a maximum rather than a minimum (Poole *et al.*, 1973).

Two explanations seem possible for the persistence of respiratory oscillations after inoculation of stationary phase cells into fresh growth medium. First, the events responsible for their generation may be components of a sequence which is normally tightly coupled to the cycle of growth and division. These cycles correspond in principle to the 'growth cycle' and the 'DNA-division cycle', respectively, proposed by Mitchison (1971). Under appropriate conditions of metabolic perturbation (in this case transition from the stationary phase to the growing state) the two sequences or cycles may be dissociated. Alternatively, the oscillations may reflect the functioning of complex biochemical regulatory mechanisms which are triggered by the onset of the stationary phase, and which are not causally related to any sequence constituting the normal cell cycle. Similar results have recently been obtained with the yeast *Candida utilis*, where the onset of the stationary phase due to depletion of the carbon source triggers an oscillation in respiration rates (D. Lloyd & R. K. Poole, unpublished results). Further characterization and comparison of the oscillations resulting from metabolic perturbation and those observed during the normal cell cycle will be necessary to distinguish between these two possibilities.

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