Fluctuations in Buoyant Density during the Cell Cycle of *Escherichia coli* K12: Significance for the Preparation of Synchronous Cultures by Age Selection

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SUMMARY

The buoyant densities of *Escherichia coli* K12 were investigated by isopycnic centrifugation in gradients of colloidal silica (Ludox) and polyvinylpyrrolidone. Bacteria from an exponential culture in a defined medium supplemented with hydrolysed casein banded at densities between 1.060 and 1.115 g ml⁻¹; the mean density was 1.081 g ml⁻¹. At the higher densities, two populations of cells were present: smaller cells were approximately twice as numerous as, and half the modal volume of, the population of larger cells. A homogeneous population of cells of intermediate volume equilibrated in the least dense region of the density band. Synchronous cultures were established by inoculating cells selected from the most or least dense regions of the band into spent growth medium. The results are consistent with a fluctuation between maximal density at cell birth and division, and minimal density near the middle of the cell cycle. In synchronous cultures prepared by continuous-flow age selection, the first division occurred after a period that was significantly shorter than the length of subsequent cell cycles. Cells selected by this procedure were of similar mean density to those in the exponential culture but were more homogeneous with respect to size. The possibility that the smallest (and densest) cells in an exponential culture are retained in the rotor, and are thus excluded from the synchronous culture, is discussed.

INTRODUCTION

There is considerable evidence that the ratio of cell mass to volume (i.e. density) fluctuates during the cell cycles of a variety of eukaryotic micro-organisms (for review, see Mitchison, 1971). Direct evidence for similar changes in the bacterial cell cycle is restricted to the measurements of single cells of *Streptococcus faecalis* by Mitchison (1961), and to the synchronization of growth of *Lineola longa* by selection of cells of discrete density classes from asynchronous populations by isopycnic gradient centrifugation (Baldwin & Wegener, 1976). However, the different patterns for accumulation of macromolecules (tacitly assumed to represent cell mass), which increases exponentially (Abbo & Pardee, 1960; Cummings, 1965), and for growth in volume, which increases linearly for much of the cycle (Kubitschek, 1968), suggest that density may fluctuate during the cell cycle of *Escherichia coli*. Indeed, cell cycle-dependent fluctuations in density or alternatively changes in cell diameter during the cycle are predicted by a theoretical model describing the growth and dimensions of *E. coli* (Zaritsky, 1975).

The present paper shows that density fluctuates during the cell cycle of *E. coli* K12. Maximum density occurs during a short period of the cycle, centred at cell division; minimum density occurs near the middle of the cycle. A new method for the preparation of

synchronous cultures by density selection, exploiting these fluctuations, is described. The possible significance of density fluctuations for the preparation of synchronous cultures by continuous flow selection of cells from an exponential culture (Lloyd *et al.*, 1975) is discussed.

METHODS

Organism and growth medium. *Escherichia coli* strain A1002 (K12 Y *mel ilv⁻ lacI⁻ metE⁻*), kindly supplied by Dr B. A. Haddock, was grown in a mineral salts medium (Poole & Haddock, 1974) containing 0.5 % (w/v) glucose and supplemented with isoleucine, valine and methionine (each at 0.002 %, w/v) and vitamin-free Casamino acids (Difco; 0.1 %, w/v).

Growth and harvesting. Starter cultures (40 ml medium in 250 ml Erlenmeyer flasks) were inoculated with a drop of a glycerol-supplemented stock culture that had been stored at -20°C . After incubation for 16 h at 37°C on a rotary shaker (200 rev. min^{-1}), a sample was diluted 400-fold into fresh medium and growth was allowed to continue for 3.25 to 4.25 h, until the extinction of the culture at 420 nm had reached 0.5 to 0.6. At this phase of growth, cell volume is fairly constant over three doublings in cell numbers (unpublished results) indicating that growth is balanced (Campbell, 1957): the mean generation time is 44 min. Organisms were rapidly and quantitatively harvested by filtration under reduced pressure through a Nuflow membrane filter (50 mm diam. $0.45\text{ }\mu\text{m}$ pore size; Oxoid). They were washed on the filter with 10 mM-potassium phosphate buffer, pH 7.4, and resuspended in the same buffer (3 to 6 ml).

Density gradient media. Ludox HS40 is a colloidal silica sol (40 %, w/v, silica; for review, see Wolff, 1975). In some experiments, the sol was brought to pH 7.4 with 2 M-HCl and diluted with 1 vol. 10 mM-potassium phosphate buffer (pH 7.4) resulting in a final concentration of 20 % (w/v) silica. In most experiments, the medium contained 3.75 % (w/v) polyvinylpyrrolidone (mol. wt 40000), approx. 16 % (w/v) silica as Ludox HS40, and sufficient 1 M-HCl to bring the pH to 7.4 to 7.6. The final density of the medium was brought to 1.098 to 1.102 g ml^{-1} by adding 3.75 % (w/v) polyvinylpyrrolidone (pH 7.5) and the medium was then filtered through a Nuflow membrane filter ($0.45\text{ }\mu\text{m}$ pore size). Gradients of Ludox were formed by exploiting the rapid self-forming properties of colloidal silica (Wolff, 1975) as described in Results.

Linear gradients of non-dialysed or dialysed Ficoll extended between densities 1.064 and 1.154 g ml^{-1} , and 1.014 and 1.137 g ml^{-1} , respectively. Metrizamide, a tri-iodinated benzamide derivative of glucose (mol. wt 789), was prepared as a 60 % (w/v) aqueous solution: linear gradients were 6 to 60 % (w/v). Dextran (mol. wt 73200) was dissolved either in water or in growth medium that contained 27.5 mM-sodium citrate (a non-utilizable carbon source) instead of glucose: gradients were 3.5 to 35 % (w/v). The pH values of these media were adjusted to 7.3 to 7.6. Linear gradients (10 ml) were prepared by layering solutions of decreasing density in MSE polycarbonate centrifuge tubes which were allowed to stand overnight before use.

Loading and fractionation of density gradients. Preformed gradients of Ficoll, Metrizamide or dextran were loaded with 2×10^9 to 5×10^9 cells in 0.5 ml buffer, and centrifuged for the times indicated in Results at 28000 rev. min^{-1} in the 6×14 ml swing-out rotor of an MSE Super Speed Centrifuge. Alternatively 1×10^{10} to 2×10^{10} cells were dispersed in 11 ml Ludox-polyvinylpyrrolidone gradient medium with one stroke of a loose-fitting hand homogenizer (Thomas, Philadelphia, U.S.A.) and the suspension was centrifuged for 64 min as described above. After centrifuging, 10-drop fractions (about 0.4 ml) were collected

through a fine glass tube immersed through the gradient from above to a level just above the mass of solidified silica that had formed at the bottom of the tube.

Synchronous division of cells following density selection. Selected fractions from Ludox-polyvinylpyrrolidone gradients were diluted with 3 ml growth medium that contained 27.5 mM-sodium citrate instead of glucose, and centrifuged for 20 min in a Microangle Bench Centrifuge. Cells from the various fractions comprising the desired density range were pooled, resuspended in 1 ml of sterile spent culture medium, and inoculated into 20 ml of the same medium, vigorously stirred and maintained at 37 °C.

Preparation of synchronous cultures by continuous flow centrifugation. An MSE Continuous Action Rotor was used essentially as described by Lloyd *et al.* (1975) but was fitted with a stelliform polypropylene insert (nominal capacity 300 ml) instead of the 'high efficiency insert' used by these authors. The rotor speed was 14 500 to 15 500 rev. min⁻¹ and the flow rate of culture from a Mariotte bottle was 160 to 170 ml min⁻¹. These conditions allowed recovery of 6 to 10 % of the original exponential culture in the rotor effluent (Poole, 1975). The first 300 ml of effluent was discarded, since the residence time of cells in this volume was longer than in the remaining culture; subsequent volumes (400 ml for determination of cell density, and 200 ml for establishing synchronous cultures) were collected into a receiver maintained at 35 to 37 °C. Synchronous cultures were grown in an open vessel vigorously aerated by magnetic stirring.

Analytical methods

Cell numbers and volumes. These were determined using a Coulter counter model Z_{BI} fitted with a probe having an aperture diameter of 30 µm. Counts are the means of at least four determinations, after correction for coincidence and for background counts due to the electrolyte alone. The electrolyte contained (g l⁻¹): NaCl (8.0), KCl (0.2), Na₂HPO₄ (1.15), KH₂PO₄ (0.2), NaN₃ (1.0), and was membrane filtered before use. A C1000 Channelyzer was connected to an ASR-33 teletypewriter by a teleprinter interface. The plotting of size distributions and their statistical analysis was performed as described by Bazin, Richards & Saunders (1977), using a CDC 6600 Computer. Ludox interfered with Coulter analysis, and so bacteria were washed twice with 3 ml 10 mM-potassium phosphate buffer (pH 7.4) during centrifugation for 20 min in a Microangle Bench Centrifuge, and finally resuspended in 0.5 ml of the same buffer. This procedure reduced the particle count due to the 1000-fold dilution of Ludox (16 %, w/v, silica)-polyvinylpyrrolidone into electrolyte to a value about one-third greater than the normal background count due to electrolyte alone.

Density measurements. These were made in the apparatus described by Miller & Gasek (1960) by allowing a 3 µl drop of the aqueous sample to sediment to equilibrium in a linear density gradient composed of kerosene and CCl₄. The gradient was calibrated with similar drops of standard KI solutions whose densities at 20 °C were determined by interpolation of data from *International Critical Tables* (1928).

Assessment of synchrony. The synchrony index (F) for the first increase in cell numbers was calculated by the method of Blumenthal & Zahler (1962). When applied to an exponentially growing culture, F = 0; when applied to a culture exhibiting perfect synchrony, F = 1.0.

Chemicals. Ficoll (lot no. 32C-0150) and dextran (clinical grade) were from Sigma. Metrizamide and Ludox HS40 were generous gifts from Nyegaard & Co, Nycoveien 2, Oslo, Norway, and Du Pont Co. (U.K.), London EC1, respectively. Odourless kerosene was from Esso Chemical. Others were generally of the highest purity commercially available.

Fig. 1. Isopycnic separation of cells from an exponentially-growing culture of *E. coli* in a mixture of Ludox and polyvinylpyrrolidone (initial density 1.099 g ml^{-1}). A total of 1.44×10^{10} cells was used; gradient formation and density equilibration were achieved by centrifugation at $6.65 \times 10^6 \text{ g min}$. Recovery was 128 %. The hatched area indicates the pellet of solidified silica at the bottom of the tube. Bottom and top refer to the orientation of the centrifuge tube during collection of fractions. Density (○), cell number (●), mean cell volume (□).

RESULTS

Isopycnic centrifugation in Ludox gradients

Gradients lacking polyvinylpyrrolidone were markedly sigmoidal in profile. Irrespective of the centrifugal force used (3.75×10^6 to $6.65 \times 10^6 \text{ g min}$) or the profile of the gradient, cells equilibrated at similar densities (average median density in five experiments, 1.113 g ml^{-1} , S.D. 0.009).

Polyvinylpyrrolidone acts on the polydisperse colloidal silica as a molecular sieve (Pertoft, 1966) enabling nearly linear self-forming gradients to be formed and protecting organisms from silica precipitation (Wolff & Pertoft, 1972). A number of trials showed that centrifugation at $6.65 \times 10^6 \text{ g min}$ of a suspension of Ludox in 3.75 % (w/v) polyvinylpyrrolidone (ρ 1.098 to 1.102 g ml^{-1}) generated a gradient that was almost linear over the range of densities exhibited by over 90 % of the cell population (Fig. 1). The percentage of the total number of cells in each density increment of the gradient is plotted against density range of the increment (0.0025 g ml^{-1}) in the histogram (Fig. 2). The density range of the band of cells is broad, 94 % of the cells equilibrating between densities 1.06 and 1.115 g ml^{-1} . In a typical experiment the mean density of the population was 1.081 g ml^{-1} whilst the modal density was 1.084 g ml^{-1} , the distribution being skewed towards higher densities. Similar distributions were observed in three experiments. These values are significantly lower than the densities observed in gradients of Ludox alone, yet continued centrifugation did not alter the density distribution. The mean cell volume decreased non-linearly with increasing density. In a control experiment, the contents of the tube, except the silica pellet, were mixed after centrifugation and the volume distribution of these cells was compared with that of the cells before centrifugation (Fig. 3a). The mean cell volume decreased slightly during centrifugation from 0.73 to $0.69 \mu\text{m}^3$. In the Figure, the two distributions have been normalized

Fig. 2. Histogram of the density distribution of cells (a total of 1.7×10^{10}) from an exponentially-growing culture of *E. coli*, and the mean volumes ($\circ$) of cells in each density class, after isopycnic centrifugation in a Ludox-polyvinylpyrrolidone gradient (initial density 1.098 g ml^{-1}). The dashed line indicates the mean volume of cells before mixing with the gradient medium.

Fig. 3. Volume distributions of cells from exponentially-growing cultures before and after isopycnic centrifugation in Ludox-polyvinylpyrrolidone gradients. Open circles ($\circ$) indicate the observed frequency of cell numbers in each of 100 size channels, determined using a Coulter counter model Z_{BI} and Channelyzer C1000 as described in Methods. Instrument settings were: aperture current, 1 mA; lower threshold, 7; upper threshold, off; 1/amplification, 0.125; count range, 1000; edit switch, on. In (a), the continuous line shows the volume distribution of cells after centrifuging and mixing the density gradient. The distribution is shifted by a volume increment of $0.06 \mu\text{m}^3$ for comparison with the volume distribution of cells before centrifuging ($\circ$). (b) Distribution of cells equilibrating at $\rho = 1.096 \text{ g ml}^{-1}$. The continuous line shows the hypothetical normal distribution of a population of modal volume $0.44 \mu\text{m}^3$. The dashed line was obtained by subtracting the continuous line values from the experimental points. (c) Distribution of cells equilibrating at $\rho = 1.078 \text{ g ml}^{-1}$. Arrows indicate modal volumes measured (b, c) or calculated (b).

with respect to their modal volume; the small volume changes resulting from exposure to Ludox and/or high centrifugal fields are independent of the volume (and thus age) of the individual cell. Cells banding at high densities (Fig. 3b) exhibited a volume distribution that was positively skewed (relative skewness 0.91). This measure of skewness has no definite

Fig. 4. Cell numbers in synchronous cultures of *E. coli* prepared by selection. Cells from an exponentially-growing culture were centrifuged to equilibrium in a Ludox-polyvinylpyrrolidone gradient. Cells of density $\rho = 1.079$ to 1.083 g ml^{-1} (b) or $\rho = 1.095$ to 1.105 g ml^{-1} (c) were inoculated into 20 ml spent growth medium. (a) Growth of cells from a gradient mixed after centrifugation. (d) Cell numbers in a synchronous culture prepared by continuous-flow age selection. An exponentially-growing culture ($4.7 \times 10^8 \text{ cells ml}^{-1}$) was passed through the continuous flow rotor (15500 rev. min^{-1} ; 170 ml min^{-1}): 7% of the original population passed into the rotor effluent. Synchrony indices (F_1) for the first complete doublings in (b), (c) and (d) were 0.70, 0.51 and 0.64 respectively. Vertical lines mark the midpoints of doublings in cell numbers. The left-hand scale refers to curves (a), (b) and (c); the right-hand scale to curve (d).

upper limit, but values as great as ± 2 indicate marked skewness (Croxtan & Cowden, 1939). Visual inspection of the distribution suggests the presence of two populations of cells of different volumes. The more clearly-resolved population (modal volume $0.44 \mu\text{m}^3$; relative frequency 100%) was assumed to be normally distributed. When the extrapolated normal distribution was subtracted from the observed distribution, the resulting population was of modal volume $0.80 \mu\text{m}^3$ and relative frequency 42%. The volumes of cells banding at low densities (Fig. 3 c) are significantly less skewed (relative skewness 0.63). The modal volume ($0.57 \mu\text{m}^3$) was intermediate between the two populations of cells banding at high density.

Sedimentation in Ficoll, dextran and Metrizamide gradients

Cells failed to band in gradients of non-dialysed or dialysed Ficoll and in aqueous dextran. All cells applied to the gradient were recovered in the pellet but their mean volume was only 85% of the applied cells. Cells equilibrated in the density range 1.12 to 1.16 g ml^{-1} (median buoyant density 1.148 g ml^{-1}) in gradients of dextran in growth medium, and shrunk by about 10%. Cells banded at higher densities (1.15 to 1.25 g ml^{-1} ; median buoyant density 1.215 g ml^{-1}) in Metrizamide gradients; the cell volume decreased by 4% during centrifugation.

Synchronous division of cells following density selection

If density varies during the cell cycle, isopycnic gradient centrifugation should resolve an asynchronous cell population into density classes, enriched with cells of a particular age (or

Fig. 5. Histogram of the density distribution of *E. coli* from the effluent of the continuous flow rotor, and the mean volumes (O) of cells in each density class after centrifugation in Ludox-polyvinylpyrrolidone (initial ρ 1.098 g ml⁻¹). An exponentially-growing culture (11×10^8 cells ml⁻¹) was passed through the continuous flow rotor (14400 rev. min⁻¹; 171 ml min⁻¹): 6% of the original population passed into the rotor effluent. The dashed line indicates the mean volume of cells before mixing with the gradient medium.

ages), which should divide synchronously when transferred to growth-promoting conditions. Cells in gradient fractions of densities 1.095 to 1.105 and 1.079 to 1.083 g ml⁻¹ respectively were collected by centrifugation and inoculated into spent growth medium to eliminate perturbations in growth caused by changes in the nutrient status of the medium. Of the cells from dense regions of the gradient, shown to contain two sub-populations, 27% of the total number exhibited a synchronous division, complete within 30 min of inoculation (Fig. 4c). A second division with a moderate degree of synchrony occurred after 90 min. A single synchronous division resulting in a precise doubling in cell numbers was observed at 50 min with cells collected from less dense regions of the gradient (Fig. 4b). This suggests that the least dense cells are intermediate in age between the two populations found at high densities. Similar results were obtained in three experiments. Figure 4(a) shows the growth of cells from a mixed gradient. A lag of 40 min preceded exponential growth with a doubling time of 78 min, longer than the mean generation time of a normal asynchronous culture.

Synchronous cultures prepared by continuous flow centrifugation

A synchronous culture, prepared by allowing the most slowly-sedimenting cells in an exponential culture to escape harvest, is shown in Fig. 4(d). The length of the first complete cycle, measured as the time between the successive midpoints in cell doublings, is 60 min, whereas the time between initiation of the culture (taken as the time of collection of half the total collected effluent) and the mid-point of the first doubling in number is only 45 min. In another experiment (not shown), this latter interval was 28 min and the duration of the first complete cycle was 50 min. In a large number of experiments the period preceding the first division was unexpectedly short.

It has been assumed (Lloyd *et al.*, 1975; Evans, 1975) that the most slowly-sedimenting cells in exponential cultures of a variety of micro-organisms are those of smallest volume. Since the density of a particle also contributes to its rate of sedimentation (de Duve,

Berthet & Beaufay, 1959), dense small cells of a population may sediment faster than other size classes and thus be retained in the rotor. Figure 5 shows the density distribution of cells in the rotor effluent in a typical experiment. The mean density (1.082 g ml^{-1}) is not significantly different from that of the exponential culture (Fig. 2) but the distribution is narrower and less skewed. That the continuous flow procedure results in the selection of cells of a particular volume (and thus age) is demonstrated by the constancy of the cell volume in the various density classes. In particular, the proportion of cells banding at densities $\rho > 1.095 \text{ g ml}^{-1}$ is decreased in comparison with the exponential culture (Fig. 2) and such cells are not significantly smaller than those throughout the density band.

DISCUSSION

The heterogeneous density distribution obtained after isopycnic density gradient centrifugation of cells from an exponentially-growing culture arises from fluctuations in density during the cell cycle. Certain aspects of the equilibration of particles in gradients of colloidal silica appear anomalous, in particular the 'density shifts' of particles (polystyrene latex beads) induced by inclusion of polymers (Pertoft, 1966; Morgenthaler & Price, 1976). In the present study, polyvinylpyrrolidone caused a significant decrease in the mean buoyant density of bacterial cells, similar to the response of thylakoid membranes of chloroplasts (Morgenthaler, Marsden & Price, 1975). It is unlikely that the inverse relationship between cell volume and density reported in this paper is an artefact due to density shifts specific for cells of a particular volume or age, since the apparent densities of particles tend to decrease progressively with decreasing size of the particles (Pertoft, 1966). Furthermore, two discrete cell populations of different volumes co-sediment to high densities. Juhos (1966) used Ludox for the separation (probably isopycnic) of *E. coli* from viruses, but did not determine the density of the band of bacteria. Mean densities observed after centrifugation in Ludox gradients lacking polyvinylpyrrolidone ($\rho 1.113 \text{ g ml}^{-1}$) are similar to those determined in Ficoll gradients by Kubitschek (1974) ($\rho 1.103$ to 1.114 g ml^{-1}). Failure of the cells to band in Ficoll gradients at densities greater than this in the present study, and the apparently high banding densities in dextran and Metrizamide may be due to dehydration of cells under hypertonic conditions, but this possibility has not been examined further.

Cyclic variations in the density of cells enclosed by a rigid wall arise from different patterns of increase in cell volume and dry mass. There is general agreement that volume increase is continuous over most of the cycle in *E. coli*, but the precise pattern is uncertain (Mitchison, 1971). The simplest model consistent with the variations in density reported here is one in which cell volume increases linearly during the entire cycle (Kubitschek, 1968) and mass increases exponentially (Fig. 6*a*). Density is maximal at times 0 and 1.0 in the cycle and falls by 6 % to a minimum at 0.44 of the cycle (Fig. 6*b*). From the age distribution of cells in an exponentially-growing culture (James, 1960; Fig. 6*c*), the predicted average volume of cells in each density class of an exponentially-growing population can be calculated. A non-linear, inverse relationship exists between density and mean volume (Fig. 6*d*). This curve closely resembles that obtained experimentally (Fig. 2). One consequence of this model is that isopycnic fractionation of an exponential culture is of limited value in studying the cell cycle by direct analysis of fractions (see Poole & Lloyd, 1973), since each density class (except the lightest) contains two populations of cells. Selection on the basis of density, however, does provide an alternative means of preparing synchronous cultures of *E. coli*.

Measurements of the mean volume and density of cells in the effluent from the continuous action rotor do not allow precise determination of the position of such cells in the cycle.

Fig. 6. Model of cell growth and predicted density fluctuations. A linear increase in cell volume (—) and an exponential increase in cell mass (---) (a) leads to a fluctuation in density (b) during the cell cycle. From the relative number of cells at each stage of the cell cycle (c), the mean volume can be calculated as a function of density (d). Cell volume, cell mass and density are in arbitrary units.

However, the finding that the smallest cells in the exponential population are the densest leads to a possible explanation of the protracted period preceding the first division in synchronous cultures of a number of micro-organisms prepared by this method (Lloyd *et al.*, 1975).

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