Which Cells are Selected from Exponential Cultures by Continuous-flow Centrifugation? The Selection of Small Cells from Cultures of *Escherichia coli* and *Schizosaccharomyces pombe* that Exhibit Minimal Density Fluctuations during their Cell Cycles

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INTRODUCTION

Buoyant density fluctuates during the cell cycles of *Escherichia coli* K12 and other bacteria (Poole, 1977*a*) and of a wide range of eukaryotic micro-organisms, including the fission yeast *Schizosaccharomyces pombe* (Mitchison, 1957; Poole & Lloyd, 1973). In *E. coli* K12 and *S. pombe* these fluctuations cause the smallest cells in an exponential culture to have sedimentation rates higher than those of certain other size classes (Poole, 1977*b*); this perhaps explains why cells selected for synchrony experiments by continuous-flow centrifugation (Lloyd *et al.*, 1975) are not always the smallest in the unfractionated culture. In view of the usefulness of the continuous-flow method, and yet the uncertainty regarding the basis of the selection, we have tested the hypothesis that density fluctuations contribute to the selection process.

METHODS

Organisms and growth conditions. *Escherichia coli* strain ML30 (NCIB 10000) was maintained and grown in the mineral salts medium used previously (Poole & Haddock, 1974) except that the amino acid supplements were omitted and the initial glucose concentration was varied as described below. Growth was in a continuous culture apparatus using a procedure designed to investigate the effects of perturbations in the entering concentration of the limiting nutrient (A. M. Pickett & M. J. Bazin, unpublished work). The fermenter was of conventional Porton design (Elsworth *et al.*, 1956); the working volume was 1 litre, the dilution rate 0.144 h^{-1} , temperature $37\text{ }^{\circ}\text{C}$, pH 7.2, and stirring speed 700 rev. min^{-1} . Sterile air was passed into the air space above the culture at 500 ml min^{-1} . Periods of 2.5 h during which the entering concentration of the growth-limiting nutrient (glucose) was 0.02% (w/v) alternated with 2.5 h periods during which the glucose concentration was 0.04% (w/v). Cells were collected over a continuous 15 h period in a refrigerated vessel, and thus consisted of bacteria collected throughout three 5 h 'feeding cycles'. During each cycle, the cell population in the chemostat fluctuated between 2.0×10^8 and 2.7×10^8 bacteria ml^{-1} . We have not measured the viability or potential for growth of the collected cells. However, their macromolecular and elemental composition is similar to previous estimates for *E. coli* (McQuillen, 1973; Bauer & Ziv, 1976), and analysis of the cell-free culture supernatant for u.v.-absorbing material suggests that no significant lysis occurs during collection (A. M. Pickett & M. J. Bazin, unpublished results).

Schizosaccharomyces pombe strain 972h⁻ was maintained and grown on the defined medium that contained 1% (w/v) glucose as carbon source (Poole *et al.*, 1973). Cultures (200 ml medium in 1 litre conical flasks) were inoculated from starter cultures that had been inoculated from an agar slope culture 5 to 6 d previously. Initial cell concentrations were 0.8×10^6 to 1.2×10^6 organisms ml^{-1} ; cultures were grown to 1.6×10^7 to 3.5×10^7 organisms ml^{-1} . Flasks were shaken at 200 rev. min^{-1} and $17\text{ }^{\circ}\text{C}$ in a rotary incubator (the ambient temperature was $13 \pm 1\text{ }^{\circ}\text{C}$).

Fractionation of cultures on isopycnic density gradients. About 1.3×10^{10} cells from 50 ml of the collected *E. coli* culture were harvested by centrifugation (17000 g at $r_{av.} \times 5\text{ min}$, $20\text{ }^{\circ}\text{C}$) and washed once under the same conditions using 50 mM-KH₂PO₄/K₂HPO₄ buffer (pH 7.5). The separation on gradients of colloidal silica and polyvinylpyrrolidone was similar to that described previously (Poole, 1977*a*).

Fig. 1. Histograms of the density distribution of *E. coli* and *S. pombe*. (a) Solid line: distribution of *E. coli* ML30, grown in a chemostat with fluctuating concentrations of glucose in the entering medium, and centrifuged to equilibrium in a colloidal silica-polyvinylpyrrolidone medium. Dashed line: distribution of *E. coli* K12 (Poole, 1977a), shown for comparison. (b) Distribution of *S. pombe*, grown at 17 °C, sonicated and centrifuged to equilibrium in a linear dextran density gradient. Mean (○) or modal (●) volumes of cells in each density class are shown.

About 1×10^9 cells from an exponentially growing culture of *S. pombe* were harvested by centrifugation (7000 *g* at $r_{av.} \times 5$ min, 20 °C), washed once with water and finally resuspended in 1 ml water. Clumped cells were disaggregated by 10 s sonication at 20 °C and a peak-to-peak amplitude of 5 μm using the titanium vibrator micro-probe (end diam. 3.2 mm) of an MSE 100 W Ultrasonic Disintegrator. A portion (0.5 ml) of this suspension was applied to a 25 to 35% (w/v) dextran gradient (linear with respect to volume; total vol. 10.8 ml) and centrifuged for 30 min at 100000 *g* ($r_{av.}$) and 20 °C in the 6 × 14 ml swing-out rotor of an MSE Super Speed 65 Centrifuge to achieve isopycnic equilibration of cells (Poole & Lloyd, 1973). Fractions (15 drops each) were collected in small test-tubes after puncturing the centrifuge-tube bottom. Dextran densities were determined using a refractometer.

Selection by continuous-flow centrifugation. The procedure used for *E. coli* was that described previously (Poole, 1977a) except that the rotor speed was 17900 rev. min⁻¹ and the culture flow rate 88 ml min⁻¹. Cells were counted and sized within 5 min of collection and were not fixed. For *S. pombe*, the rotor speed was 6100 rev. min⁻¹ and the culture flow rate 450 ml min⁻¹. Small samples of both the exponential culture and the rotor effluent were fixed by adding formaldehyde to a final concentration of 2% (w/v). The total centrifugal force is expressed as *g* (at half-maximal radius) × mean residence time of the culture in the rotor (min). Mean residence time is given by rotor volume (ml)/flow rate (ml min⁻¹).

Cell numbers and volumes. These were determined as described previously (Poole, 1977a, b). Samples of *S. pombe* were sonicated as described above before counting, to eliminate the clumps that form during growth at 17 °C. Estimates of the proportion of cells existing as unseparated pairs (Mitchison, 1970) in unsonicated samples were made by phase-contrast microscopy of 300 to 800 randomly selected cells. *Escherichia coli* was first fixed with formaldehyde (final concn 2%) and stained with methylene blue.

Chemicals. Dextran (clinical grade; mol. wt 73200) was from Sigma.

RESULTS AND DISCUSSION

Escherichia coli ML30, grown under conditions of fluctuating glucose supply, banded in a very narrow density range in colloidal silica-polyvinylpyrrolidone gradients (Fig. 1a); 87% of the cells banded between densities 1.077 and 1.087 g ml⁻¹, in contrast to strain K12

Fig. 2. Volume distributions of cells in the initial population (●) and in the effluent obtained from continuous-flow centrifugation of the population (○). (a) An overnight collection of *E. coli* ML30 (2.68×10^8 cells ml^{-1}) was passed through the continuous action rotor (17900 rev. min^{-1}) at a flow rate of 88 ml min^{-1} (5.11×10^4 g-min); 14.5 % of the original population passed into the effluent. Cell volumes and numbers were determined using a Coulter counter ZBI and Channelyzer C1000 with the following settings: aperture current, 0.5; lower threshold, 4; upper threshold, off; 1/amplification, 0.5; count range, 1000; edit switch, on. (b) An exponential culture of *S. pombe* grown at 17 °C (2.27×10^7 cells ml^{-1}) was passed through the continuous action rotor (6100 rev. min^{-1}) at a flow rate of 450 ml min^{-1} (1.16×10^8 g-min); 6.3 % of the original population passed into the effluent. Samples of both the exponential culture and the effluent were sonicated before counting and sizing of cells. Instrument settings were: aperture current, 1; lower threshold, 2; upper threshold, off; 1/amplification, 0.5; count range, 1000; edit switch, on. The two distributions in each of (a) and (b) have been normalized giving equal areas under the curves.

(Poole, 1977a; shown for comparison in Fig. 1a). Mean volume decreased by only 11 % as the density increased from 1.065 to 1.092 g ml^{-1} ; there were no measurable changes in modal volume. The volume distribution of cells in each density class (not shown) was unimodal and skewed towards small volumes, resembling closely the volume distribution of the unfractionated culture. Figure 1(b) confirms the findings of Mitchison *et al.* (1963); *S. pombe* grown at 17 °C (mean generation time 11.5 h in our experiments) exhibited a narrow density distribution. All cells banded within a density increment of 0.035 g ml^{-1} , exhibiting only a 10 % change in volume over this range, whilst cells grown at 30 °C banded within an increment of 0.08 g ml^{-1} , and mean cell volume changed almost twofold over this range (Poole & Lloyd, 1973). Again, the volume distribution curve for each density class was similar to that of the exponential culture.

Continuous-flow centrifugation of *E. coli* ML30 (Fig. 2a) selected a homogeneous population of cells of significantly smaller volume (mean 0.236 μm^3) than those in the exponential culture (mean 0.396 μm^3). For *S. pombe*, the difference was less striking (Fig. 2b). The selected population (mean volume 22.2 μm^3), although having a somewhat skewed volume distribution, was enriched in cells smaller than the modal class in a sonicated sample of the unfractionated culture (mean volume 28.5 μm^3).

Extensive clumping occurred in cultures of *S. pombe* grown at 17 °C; only about 25 % of the total number of cells in the culture were present as single cells, whilst in cultures grown at 30 °C this proportion was about 88 %. This clumping explains the heterogeneity of the selected cells shown in Fig. 2(b), compared with the fractionation of *E. coli* in Fig. 2(a). The conditions chosen for centrifugation allowed recovery of about 6 % of the total number of cells in the exponential culture after sonication. Since only 25 % of this total are present as single cells, the selected cells represent a large proportion (about 25 %) of the single cells and thus a rather heterogeneous population. That the selection process excludes doublets

and large aggregates is shown by the ineffectiveness of sonication in increasing the number of particles counted in the effluent (results not shown). For *E. coli* $\kappa 12$ and *E. coli* ML30, less than 10% of the total cell number in an exponential culture were present as doublets and none as larger aggregates.

The results of selection are quite different from those published previously: continuous-flow centrifugation of *E. coli* $\kappa 12$ selected cells of a volume similar to the mean in the exponential culture (Poole, 1977a) whilst for *S. pombe* (grown at 30 °C) the cells in the rotor effluent were of a greater mean volume than those in the original culture (Poole, 1977b). These differences cannot be attributed merely to the different degrees of clumping in the previous and present results. For *E. coli*, the small proportion of cells present as doublets would not significantly alter the apparent mean volume of cells in the exponential culture; for *S. pombe*, comparisons are made between cells in sonicated samples (from 17 °C cultures) or else the degree of clumping is too low to alter apparent mean cell volumes significantly (30 °C). We therefore attribute the observed differences to the occurrence of cell density fluctuations (and their contribution to S-values) in earlier experiments, and suggest that it should not be assumed that cells selected by this method are always the smallest in the original culture. Clearly, growth conditions, species and perhaps strain differences can markedly affect the results of the selection. We have not grown *E. coli* $\kappa 12$ under the chemostat conditions used here for strain ML30, and so are unable to state whether the differences between previous results and those in this paper are due to strain difference only. Such strain differences are known to occur; the density of *E. coli* B/r varies by only 0.008 g ml⁻¹ (equivalent to about 0.7% of the mean) during the cell cycle (Koch & Blumberg, 1976).

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