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Synchronization of Cell Division in Microorganisms by Percoll Gradients

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We describe a method for obtaining synchronously dividing cells of bacteria (*Escherichia coli* B and K-12 and *Bacillus subtilis* 168) and fission yeasts (*Schizosaccharomyces pombe*) by the use of Percoll density gradients.

Several synchronization techniques have been developed for the study of sequential changes during the division cycle of bacteria. The methods consist of induction of synchrony by specific environmental shifts (such as temperature [3] or nutrition [11]) or by separation of newly divided cells by filtration (4) or by centrifugation (6). One of the best methods for selection of newly divided cells is the membrane elution technique developed by Helmstetter (2). The method consists of selectively eluting newly divided cells from a randomly growing culture adsorbed to an inverted Millipore membrane filter. The advantage of this technique is that the physiological perturbations are minimal. The major disadvantages are the low yield of cells, which renders conventional biochemical methods inapplicable, and the fact that satisfactory synchrony is obtained only with strains B/r (2) and B (10) of *Escherchia coli.* Since many cell division mutants were isolated in *E. coli* K-12, which is not easily synchronized by the membrane elution technique (13), we looked for another method for obtaining newly divided cells which will be more general and also applicable for *E. coli* K-12. Our results with sucrose gradients (1, 6) were unsatisfactory due to an inhibitory effect on the cells and to poor synchrony, and Ludox gradients, which have also been used (9), are toxic and very viscous.

We describe here separation of newly divided cells by the use of Percoll gradients. Percoll has been previously used in mammalian tissue cultures for separation of cells and cell organelles (7) and for enrichment of viable cells. Percoll is colloidal silica which is coated with polyvinylpyrrolidone to protect the cells from toxic effects. One of the major advantages of Percoll is that there is no free polymer in the solution, and therefore the viscosity is relatively low, even at high densities, and the osmotic properties are good. It was found by Pertoft et al. (8) that mammalian cells are less inhibited by Percoll than by other density gradients such as Ficoll, metrizamide, and sodium metrizoate.

We studied the effect of Percoll on several microorganisms and its possible use for obtaining synchronously dividing cultures. The results presented in this communication indicate that synchronized cultures can be obtained in bacteria (*E. coli* B and K-12 and *Bacillus subtilis* 168) and in yeasts (*Schizosaccharomyces pombe*) by selecting newly divided cells from an exponentially growing culture.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* K-12 DG-76 (F^- *thyA47 leu-6 dra-3 str-153*; kindly supplied by B. Bachmann) and *E. coli* B (*thyA ilvA*; obtained in our laboratory by mutagenesis of *E. coli* B) were grown in a minimal salts medium (2) containing 0.2% glucose and supplemented, when required, with 20 μg of thymine, 50 μg of the required amino acid, and 1 μg of thiamine per ml.

B. subtilis 168 (*try*) was grown on minimal salts medium (14) containing 0.5% glucose and 100 μg of sodium L-glutamate per ml and supplemented with 50 μg of tryptophan per ml.

S. pombe (*met4-3*), derived from the haploid wild-type strain 972 with mating type h^- (kindly provided by U. Leupold and P. Munz) was grown in EMM2 medium (5) supplemented with 50 μg of methionine per ml.

Exponentially growing cultures were obtained by inoculating 10 to 100 ml of medium and incubating in a shaking water bath. All cultures were grown at 32°C except *B. subtilis,* which was grown at 37°C. The experiment was started after about 16 h of exponential growth when the culture contained not more than 10^8 bacteria or 1.2×10^6 yeasts per ml.

Percoll gradients. Preformed gradients of Percoll (Pharmacia Fine Chemicals) were generated with a gradient maker. Linear continuous gradients of 9 ml, "0 to 100%," were prepared in growth medium according to the manufacturer's instructions. Density was determined by marker beads (Pharmacia Fine Chemicals).

For synchronization of bacteria exponentially growing cultures were concentrated by centrifugation, and

4×10^9 cells were loaded on top of the gradients and centrifuged in a Sorvall RC2-B centrifuge for 15 min at 24°C at 1,200 rpm in an HB-4 swing-out rotor. After centrifugation, 500-μl fractions were collected from the top of the gradient (about 18 fractions). When working with yeasts, about 1.2×10^9 cells were loaded and centrifuged for 2.5 min as described above.

Measurement of cell concentration. Cell counts were obtained by the use of a Cytograph (model 6301, Bio/Physics System, Inc.) for bacteria and a Coulter Counter model B, 70-μm orifice, for yeasts. Bacteria were fixed with 0.2% formaldehyde, and yeasts were fixed with formaldehyde-acetic acid-70% ethanol (1:1: 18).

Measurement of cell length. Analysis of cell length was obtained using electron microscopy of cells prepared by agar filtration according to the method of Woldringh et al. (15). For each size distribution at least 300 cells were measured.

Protein synthesis. Protein synthesis was determined by incorporation of [^{14}C]leucine into trichloroacetic acid-precipitable material. [^{14}C]leucine (59.5 mCi/mmol) was added to the medium in which cells were diluted after centrifugation in Percoll, to a final concentration of 1 μCi (50 μg) per ml. The culture was incubated at 32°C, and 0.1-ml samples were removed at 10-min intervals and precipitated with 10% trichloroacetic acid on Whatman 3mm filters. The filters were washed with 10% and 5% trichloroacetic acid, ethanol, ethanol-ether (1:1, vol/vol), and ether. They were then dried and counted in toluene in a Tricarb scintillation counter (Packard).

RESULTS AND DISCUSSION

Selection of newly divided cells of *E. coli* B by density gradients. To examine the distribution pattern of cells of various ages in Percoll density gradients, we started with synchronously dividing cultures of known cell ages. Such cultures could be obtained in *E. coli* B by using the membrane elution technique (2). A population of newly divided cells eluted off the membrane by this method will divide synchronously (Fig. 1), and it is possible to obtain from it cell populations of known ages. Figure 2 illustrates the length distribution of cells eluted from the membrane after 0 and 30 min of growth, and Fig. 3 illustrates the banding properties of such cells in Percoll gradients. The results indicate that the younger, smaller cells band at a higher density than the cells which are more advanced in the cycle.

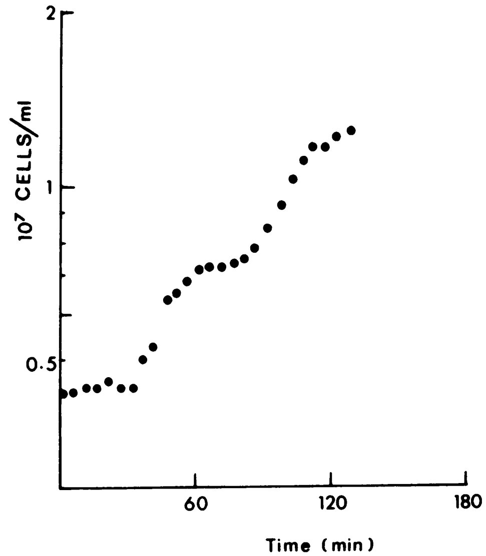

FIG. 1. *Growth of newly divided cells obtained by the membrane elution technique. Newly divided cells of E. coli B were obtained as previously described (2). The cells were incubated at 32°C, and samples were removed for counting at 5-min intervals.*

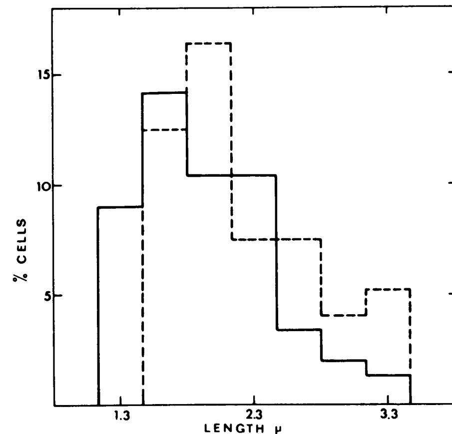

FIG. 2. *Length distribution of synchronously dividing cells of E. coli B obtained by the membrane elution technique. Cells were obtained as described for Fig. 1 and fixed with OsO_4 (0.2%) after 0 min (——) and 30 min (-----) of growth. Length distribution was determined as described in the text.*

This finding suggested that it should be possible to use Percoll gradients to select newly divided cells from an exponentially growing culture. Therefore, cultures were layered on gradients and centrifuged; the most dense fraction that still contained bacteria (fraction 13) was diluted in growth medium and incubated at 32°C, and cell number was followed. The cells divided synchronously for several generations (Fig. 4), and the timing of the first division indicated that the initial cell population obtained from the gradients consisted of very young, newly divided cells. It should be noted that the cells were not inhibited by the centrifugation in Percoll as can be seen by the immediate uptake of [^{14}C]leucine into protein (Fig. 5).

Synchronous division of newly divided

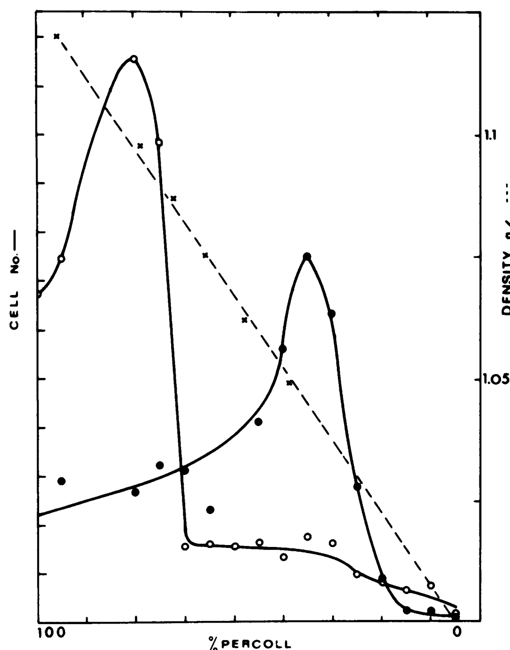

FIG. 3. *Sedimentation pattern of cells of a synchronously dividing culture of E. coli B on Percoll density gradients. Cells at the age of 0 min (○) and 30 min (●) were obtained as described for Fig. 2. About 10^8 cells were layered on 9-ml, 0 to 100% Percoll gradients, and centrifuged for 15 min at 1,200 rpm at 24°C. Fractions of 500 µl were collected. Cell number (——) was determined in the cytograph, and density (-----) was determined by marker beads.*

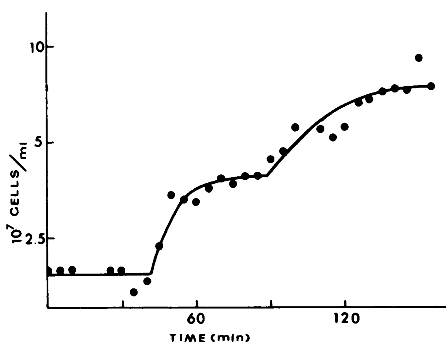

FIG. 4. *Cell division in newly divided cells of E. coli B obtained from Percoll density gradients. A sample of 4 × 10^9 cells of an exponentially growing culture of E. coli B was layered on a 9-ml, 0 to 100% Percoll gradient and centrifuged for 15 min at 1,200 rpm at 24°C, and 500-µl fractions are collected from the top. The most dense fraction that contained bacteria (no. 13) was diluted in 12 ml of prewarmed growth medium and incubated at 32°C. Samples were removed at 5-min intervals for determining cell concentration.*

cells of *E. coli* K-12, *B. subtilis* 168, and *S. pombe*. Percoll density gradients were also used for obtaining synchronously dividing cultures in *E. coli* K-12. In this strain, as in *E. coli* B, the most dense fraction of bacteria contained newly divided cells, as can be seen from their length distribution (Fig. 6) and division pattern (Fig. 7). Synchronously dividing cultures could also be obtained by this method from exponentially growing cultures of *B. subtilis* 168 (Fig. 8). Although synchrony was poorer with *B. subtilis* than with *E. coli*, it was much better than what

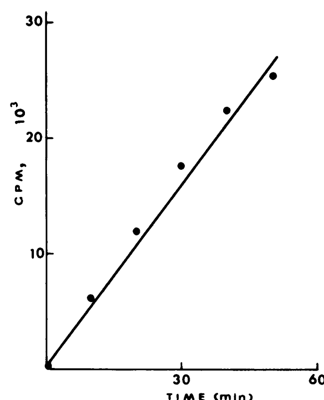

FIG. 5. *Protein synthesis in cells of E. coli B after centrifugation in Percoll density gradients. Protein synthesis was determined by incorporation of [^{14}C]leucine as described in the text.*

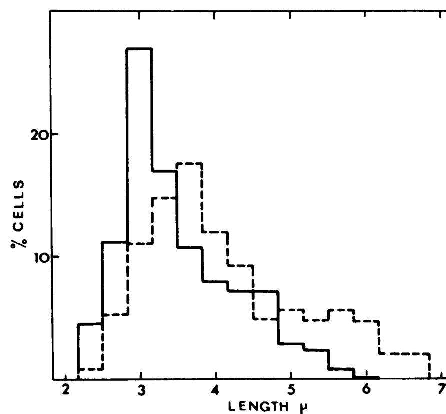

FIG. 6. *Cell length distribution of cells from an exponentially growing culture of E. coli K-12 after centrifugation in Percoll density gradients. Cells of strain DG-76 of E. coli K-12 were obtained from a Percoll gradient and treated as described in Fig. 4. Cells from the most dense fraction which contained bacteria (no. 13) (——) and cells from an exponentially growing culture (-----) were fixed and analyzed as described for Fig. 2.*

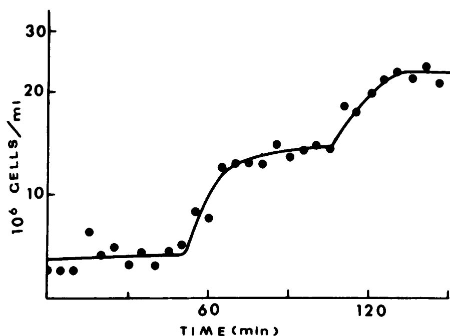

FIG. 7. *Cell growth of newly divided cells of E. coli K-12 obtained from Percoll density gradients. Cells of strain D6-76 of E. coli K-12 were obtained from a Percoll gradient and treated as described in Fig. 4. Samples were removed at 5-min intervals for determining cell concentration.*

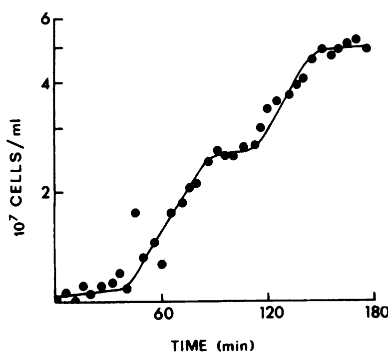

FIG. 8. *Cell growth of newly divided cells of B. subtilis 168 obtained from Percoll density gradients. Cells of B. subtilis 168 were obtained from a Percoll gradient and treated as described in Fig. 4. Samples were removed at 5-min intervals for determining cell concentration.*

we obtained by the use of other methods. The synchronization index calculated (12, 16) from the experiments presented in this communication was 0.55 for *B. subtilis* 168, as compared with 0.7 for *E. coli* B and 0.8 for *E. coli* K-12.

The fission yeast *S. pombe* could also be synchronized on Percoll gradients. However, since these cells formed a narrow band under the conditions used for bacteria, synchrony was obtained by taking the top part after a rather short centrifugation (Fig. 9).

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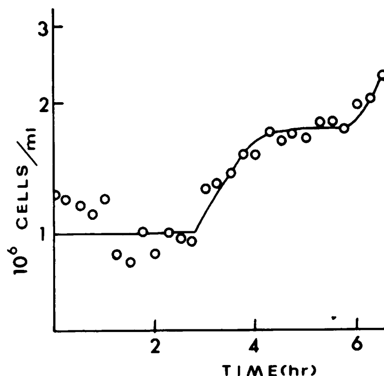

FIG. 9. *Cell division of newly divided cells of S. pombe obtained by Percoll gradients. A sample of 1.2 × 10⁷ cells of S. pombe from an exponentially growing culture was layered on top of a Percoll gradient. After centrifugation for 2.5 min at 1,000 rpm, the top layer of the band was removed, suspended in prewarmed growth medium, and incubated at 32°C. Samples were removed at 15-min intervals for cell counting.*

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