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Improved Bacterial Baby Machine: Application to *Escherichia coli* K-12

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Exponentially growing derivatives of *Escherichia coli* K-12 were immobilized onto the surfaces of nitrocellulose membrane filters which had been coated with poly-D-lysine. The cells attached firmly to the surfaces, and when flushed with culture medium, the immobilized cells continued to divide and newborn cells were released into the effluent. Cell cycle parameters were examined with the technique, and it was found that K-12 derivatives possessed differing values for interdivision times, *C*, *D*, and average cell sizes when grown in the same culture media. It was also found that the cells released from immobilized populations of one culture consisted of two predominant size classes: newborn cells of unit size with single nucleoids and newborn cells of double this unit size. The results demonstrated that K-12 derivatives can be used in the baby machine culture technique to examine all aspects of the cell cycle of this organism. Furthermore, the yield of newborn cells was about fivefold greater than that obtained previously with cultures of strain B/r immobilized onto uncoated membranes.

In the baby machine culture technique, cells are attached to a surface such that each time a cell divides, one newborn progeny cell remains attached and the other one is released. This culture technique, which employs a nitrocellulose membrane filter as the binding surface (8, 11), has been used to define cell cycle properties of *Escherichia coli* B/r (4, 12). Since newborn cells are released from the immobilized culture continuously, samples of the effluent can be collected and will grow synchronously for use in cell cycle research. However, the most common application of the technique has utilized a retrospective approach to study the cell cycle. As an example, the relationship between chromosome replication and the division cycle was determined by pulse-labeling an exponentially growing culture of cells with radioactive thymidine, immobilizing the culture in a baby machine, and measuring the radioactivity in the newborn cells shed continuously from the culture (5, 7).

The primary reason this technique proved successful for investigations on the cell cycle is that it minimized problems associated with disturbance of the physiological state of the cells (4). One of the major disadvantages of the technique, however, has been that it was not readily applicable, as described, to other *E. coli* strains, particularly K-12. For the technique to have widespread utility, the cells initially filtered onto the membrane surface must adhere firmly, and the unattached daughter cells produced by a division must be shed unimpeded from the surface. In attempts to accomplish this with strain K-12, membrane filters of different compositions have been tested, and some success with polyvinyl chloride membranes has been reported (5). However, general applicability to the many K-12 derivatives has not been developed, and, as a consequence, the technique has not been used routinely with any *E. coli* strains other than B/r. In this article, we report a modification of the procedure which enables the use of K-12 strains and possibly many other bacterial strains and cellular species. The modification entailed coating the nitrocellulose membrane with an adhesive prior to attachment of the bacterial cells.

MATERIALS AND METHODS

Bacteria and growth conditions. The strains employed were *E. coli* B/r F26 (*thyA his*) (9), K-12 wild type (F^+) (CGSC 5073), K-12 C600 (*leuB6 thr-1 fhuA21 lacY1 supE44 rfbD1 glpR200 thi-1* λ^-) (CGSC 3004), K-12 C600(pLG338) (14), K-12 M182 [Δ(*lacIPOZYA*)*X74 galU galK* $StrA^r$] (3), and K-12 AB1157 [*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xtl-5 mtl-1 argE3 thi-1 rac*$^-$ λ^-] (2). Plasmid pLG338 is a derivative of pSC101 carrying determinants for resistance to kanamycin and tetracycline. Cultures were grown in minimal salts medium (7) supplemented with 0.1% glycerol, 0.1% glucose, or 0.1% glucose plus 0.2% Casamino Acids (Difco Laboratories). When necessary, thymine was added at 10 μg/ml and amino acids and thiamine were added at 25 μg/ml. For each experiment, 100 ml of minimal medium was inoculated with bacteria and incubated for at least 18 h at 37°C in a shaking water bath until the culture was growing exponentially at a concentration between 2×10^7 and 5×10^7 cells per ml. In some experiments, the cells were grown into early stationary phase, diluted approximately 1:1,000 in 100 ml of fresh minimal medium, and incubated further until the cells were growing exponentially at the desired concentration. Bacterial concentrations were determined with a model ZB Coulter electronic particle counter. Cell size distributions were determined with a Coulter model 256 channelizer connected to a Coulter X-Y recorder. The A_{450}'s of the cultures were measured with a Milton Roy spectrophotometer.

Modified baby machine technique. The membrane filter holder used in the technique has been described previously in detail (8, 10). A type GS, 142-mm-diameter, 0.22-μm-pore-size nitrocellulose membrane filter (Millipore) was clamped into the holder. Before immobilization of the cells, a 50-ml solution containing 5 μg of poly-D-lysine per ml (Sigma; molecular mass, 30 to 70 kDa) in Dulbecco's phos-

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phate-buffered saline (GIBCO) was drawn through the membrane filter under vacuum at a rate of approximately 1 ml/s. The membrane was then washed by drawing 100 ml of distilled water rapidly through the membrane. The cells were immobilized onto the surface by filtering 100 ml of an exponentially growing culture in minimal medium through the membrane. Filtration was stopped before all of the culture medium had been drawn through the filter, to avoid drying the cells, and the remainder of approximately 10 ml was poured off. In experiments involving radioactive labeling, the attached cells were then washed by drawing 100 ml of fresh medium (containing 100 μg of thymidine per ml) through the filter. The filter holder was then inverted, and fresh medium was poured into the upper part of the filter holder (ca. 200 ml). The upper chamber of the holder was connected to a peristaltic pump. The pump was operated at a rate of 15 ml/min for 2 min to remove unattached cells, and then the rate was reduced to 2 ml/min. Consecutive samples of the effluent were collected and analyzed.

Cell cycle analyses. When the cultures had reached the desired concentrations, they were exposed to either 2 μCi of [*methyl*-^{3}H]thymidine per ml (NEN; 85 Ci/mmol) or 0.04 μCi of [2-^{14}C]thymidine per ml (ICN Radiochemicals; 51 mCi/mmol) for 4 min. Unlabeled thymidine (final concentration, 100 μg/ml) was added at the end of the labeling period, and the cells were introduced into a baby machine. Consecutive 4-min samples of the effluent were collected. A 0.5-ml portion was removed from each sample and diluted in 9.5 ml of Isoton II (Coulter) for measurement of cell concentration, and the remainder of the sample was added to ice-cold trichloroacetic acid (final concentration, 5%). Radioactivity incorporated per cell was determined by filtering the samples onto 25-mm-diameter glass fiber filters (Whatman) and then by successively washing each filter with equal volumes of cold 5% trichloroacetic acid, water, and 95% ethanol. After drying, the filters were placed in Ecoscint O (National Diagnostics) and counted in a Beckman LS 5000TD liquid scintillation counter.

RESULTS

Newborn cells eluted from baby machines. Figure 1 shows the concentrations of cells in consecutive samples of the effluent from baby machines with *E. coli* K-12 wild type and M182 immobilized on poly-D-lysine-coated and uncoated membrane filters. In the absence of polylysine coating, the concentration of cells in the effluent began to decline after 40 to 60 min and then decreased continuously. The decrease in cell concentration with uncoated membranes was seen with all K-12 strains and often resulted in release from the membrane of essentially all of the initially bound cells. This detachment of cells may have been related to the presence of fimbriae and/or flagella on the cell surface because of weaker attachment or active detachment of motile cells. The more motile M182 became detached more rapidly than the less actively motile K-12 wild type. The strain B/r derivatives used in the past were not motile. In the presence of the coating, cells were firmly attached and the concentration of cells in the effluent increased in a manner similar to that seen with strain B/r (7). The yield of newborn K-12 wild-type cells from the instrument was approximately fivefold greater than that obtained previously with strain B/r bound to uncoated nitrocellulose membranes (8).

A comparison of K-12 C600(pLG338) cell size distributions in the effluent from a baby machine with those in an exponential-phase culture is shown in Fig. 2. The majority of

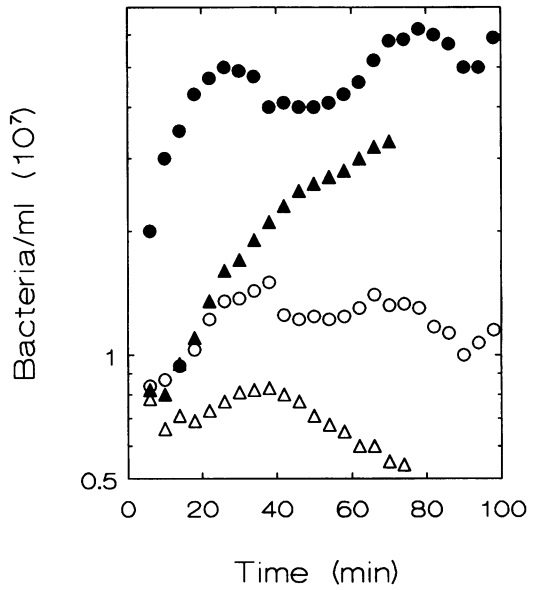

FIG. 1. Cell concentrations in consecutive samples of the effluent from membrane-bound *E. coli* K-12 cultures. In each experiment, a 100-ml culture in glucose minimal medium was filtered onto the surface of a nitrocellulose membrane filter with (solid symbols) or without (open symbols) a poly-D-lysine coating. Symbols for cells and the concentrations applied to the membranes: ●, wild type at 4 × 10^7 cells per ml; ○, wild type at 3.8 × 10^7 cells per ml; ▲, M182 at 2.6 × 10^7 cells per ml; △, M182 at 2.0 × 10^7 cells per ml

cells in the effluent resided in a narrow distribution positioned at the ascending portion (smallest size) of the exponential-phase culture, as expected for release of daughter cells at division. The relative size distribution of the small newborn cells was typical for all of the K-12 derivatives examined. The unusual aspect of the distribution was the appearance of a second peak positioned at the descending

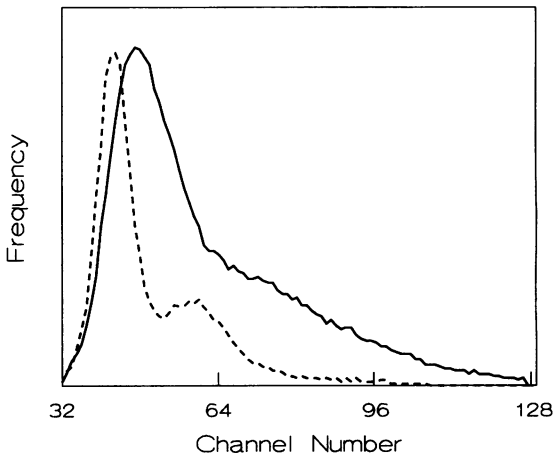

FIG. 2. Size distributions of exponential-phase and baby machine-generated cells. Size distributions in a sample from an exponential-phase, glucose-grown culture of *E. coli* C600(pLG338) containing 4 × 10^7 cells per ml (——) and in a sample collected from the effluent of the same culture after it had been bound to a poly-D-lysine-coated membrane (– – –) are shown.

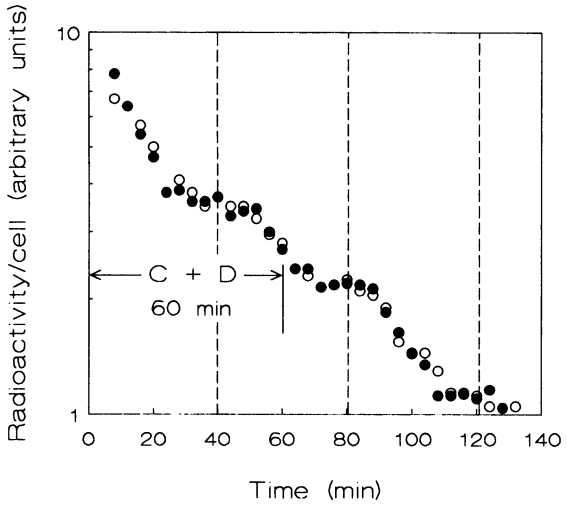

FIG. 3. Radioactivity per cell in the effluent from cultures of *E. coli* B/r F26 and K-12 wild type bound to polylysine-coated membranes. Cells growing in glucose minimal medium were pulse-labeled with radioactive thymidine for 4 min, and radioactivities per cell in the effluent from baby machine experiments are shown. Symbols: ●, wild type; ○, B/rF. The interrupted vertical lines indicate generations of elution.

portion of the distribution for exponential-phase cells, which would correspond to cells of double the size of the small newborn cells. These data, in addition to microscopic examination of the cells in the effluent, indicate that two classes of cells were released from the surface: newborn cells of unit size and cells of approximately twice the unit size.

Chromosome replication during the division cycle. The relationships between chromosome replication and the division cycle for strain K-12 derivatives were examined as described previously for strain B/r (7). In brief, 100-ml cultures growing exponentially were pulse-labeled with radioactive thymidine for 4 min and then bound to polylysine-coated membrane filters. Figure 3 shows the radioactivity per effluent cell for glucose-grown *E. coli* K-12 wild type compared to that for B/r F26. The vertical interrupted lines indicate approximate generations of elution. The generation times (τ) were determined by the time interval between inflections of the concentration of cells in the effluent, as described previously (7). Since the cells that eluted during each generation were progeny of the oldest through the youngest cells initially bound to the membrane, the rate of thymidine incorporation during the division cycle is read from right to left in each generation (Fig. 3). The results for K-12 wild type and B/r F26 were identical, with the rate of incorporation increasing in the middle of the division cycle. This increase in incorporation corresponds to initiation of a round of chromosome replication (5). The time between the start of a round of replication and the division after completion of that round, $C + D$ (in minutes), is given in Fig. 3 as the time between the start of elution and the mean time of the step in radioactivity per cell in the second generation of elution. In both strains, $C + D$ was equal to 60 min in these experiments.

Most of the K-12 derivatives examined to date possessed growth rates and/or C and D values that differed from those of K-12 wild type and B/r F26. K-12 C600, which was derived from K-12 wild type after three separate exposures to X-rays and UV irradiation (2), possessed C and D values indistinguishable from those of the wild type but grew more slowly in identical culture medium (Table 1). C600(pLG338), on the other hand, was found to possess longer C, D, and τ values at all growth rates. Figure 4 shows radioactivity per effluent cell for experiments performed with this derivative in minimal medium with glycerol, glucose, and glucose plus Casamino Acids. The generation times on the membrane in these three growth media were 115, 75, and 38 min, respec-

TABLE 1. Cell cycle parameters in glucose-grown cultures

Strain	τ (min)[a]	$C + D$ (min)[b]	C (min)[c]	D (min)[c]	$A_{450}/10^8$ cells[d]
B/r F26	40	60	42	18	0.182
K-12 wild type	40	60	40	20	0.225
K-12 C600	55	61	43	18	0.198
K-12 M182	54	61	41	20	0.206
K-12 AB1157	55	76	52	24	0.194
K-12 C600(pLG338)	75	82	56	26	0.220

[a] Generation times were determined for the membrane-bound cultures as the time between peaks in the concentration of cells in the effluent (7).
[b] Data represent the average values from at least two experiments of the type shown in Fig. 1 and 2.
[c] D was estimated as the time for continuation of division in the presence of 200 μg of chloramphenicol per ml (for references to the method, see reference 9). C is taken from $(C + D) - D$.
[d] Cell concentrations were determined with a Coulter electronic particle counter calibrated for CFU.

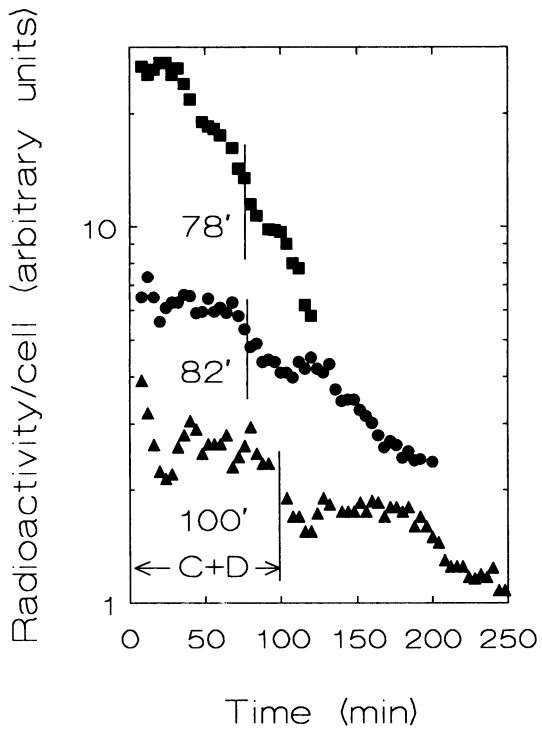

FIG. 4. Radioactivity per cell in the effluent from membrane-bound cultures of *E. coli* K-12 C600(pLG338) growing at different rates. Cells growing in minimal medium with glucose plus Casamino Acids (■), glucose (●), or glycerol (▲) were exposed to radioactive thymidine for 4 min. Measurements of $C + D$ are indicated for each growth rate.

tively, as opposed to 50, 40, and 26 min for strain B/r F26 in the same media (9). The values for $C + D$ are shown in Fig. 4 and were longer than those for the other derivatives.

Cell cycle data for several derivatives grown in glucose minimal medium are summarized in Table 1. Average mass per cell, as determined by A_{450}, was larger for the K-12 derivatives than for B/r. Although there was a somewhat higher level of cell filamentation in the K-12 populations, the differences in average sizes were not entirely explainable on this basis because similar differences were seen in measurements of cell size distributions of newborn cells collected from baby machines (data not shown). Since K-12 wild type was larger than B/r F26, but possessed the same values for C, D, and τ, mass at initiation of chromosome replication was also larger. K-12 C600, M182, and AB1157 were smaller than K-12 wild type when grown in glucose minimal medium but about the same size when grown in medium supplemented to achieve the same growth rate. K-12 C600 (pLG338) was approximately the same size as K-12 wild type in glucose minimal medium even though the generation time was almost twice as long.

DISCUSSION

It is anticipated that the modification of the baby machine procedure reported here is generally applicable to all strains of *E. coli* and other bacterial species. The coating of the nitrocellulose membrane with an adhesive to ensure attachment of cell populations was the principal requirement for success with the procedure. In the case of the poly-D-lysine coating reported here, the concentration of cells loaded onto the membrane was an important consideration. Most of the cells filtered onto the coated membrane became attached, and the membrane became saturated with 5×10^9 cells. Therefore, the concentration of cells in the 100-ml culture filtered onto the membrane could not exceed 5×10^7 cells per ml. Lower concentrations of cells were used in some experiments, but in these instances both daughter cells remained attached during some cell divisions in the early stages of elution, as indicated for strain M182 in Fig. 1. This phenomenon did not adversely affect the results for cell cycle experiments but would be a disadvantage for some applications such as studies on segregation of components between daughter cells.

As is the case with baby machine experiments with *E. coli* B/r on uncoated nitrocellulose membranes, the composition of the culture medium can affect the homogeneity of release of newborn cells from the surface of coated membranes by interfering with attachment. A rich medium such as nutrient broth could not be used, and the selective release of newborn cells was slightly poorer with media containing Casamino Acids than those without, presumably because of interference in cell adhesion caused by the presence of charged amino acids. The concentration of poly-D-lysine in the solution drawn onto the membrane was not critically important and could be varied 10-fold higher or lower than the recommended 5 μg/ml with little effect on the results.

The observation that cells released from membrane-attached cultures of K-12 C600(pLG338) consisted of two distinct size classes was unexpected. We had considered that one of the reasons K-12 derivatives had not been used routinely in the technique was due to the higher frequency of cell filaments in K-12 derivatives than in strain B/r. Formation of such filaments would obviate use of the technique for cell cycle studies if the filamentation resulted in cell divisions at random sizes (or ages). The results of the current experiments suggest that this is not a major concern. Even in a culture such as C600(pLG338) which possesses a significant proportion of cell filaments, the cells may continue to divide at regular intervals to form some larger newborn cells which are multiples of unit size. The implications of this finding as regards the timing and placement of division sites, and the relationship to current models for the control of this process, are under investigation. Some K-12 derivatives may have a broader size distribution at division, particularly those that possess mutations in division-associated genes, thus limiting their use with the technique. For the same reason, achievement of balanced growth prior to cell cycle analyses with the technique is also an important consideration (13).

From the findings reported here and previously (1, 9), it is evident that K-12 derivatives may possess differing values for C, D, τ, and cell size. It is particularly interesting that maintenance of the pSC101 derivative, pLG338, in K-12 C600 resulted in larger values for both C and D. In our previous studies with plasmid-containing *E. coli* B/r, we had not detected alterations of cell cycle properties of any kind except when the cells harbored composite plasmids containing *oriC* and an origin from a high-copy-number plasmid such as pBR322. Furthermore, introduction of pLG338 into B/r F26 did not alter the growth or cell cycle properties of this strain (data not shown). When present in C600, the plasmid may compete for a component involved in chromosome replication, but it is difficult at present to explain how that competition could simultaneously affect C, D, cell size, and τ. Efforts to explain this observation are in progress.

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