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Cell Cycle Parameters of Slowly Growing *Escherichia coli* B/r Studied by Flow Cytometry

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The cell cycle kinetics of *Escherichia coli* B/r A and B/r K cells were studied by flow cytometry. Three-dimensional histograms of cell cultures show the number of cells as a function of cellular DNA and protein contents and give detailed pictures of the cell cycle distribution with regard to these parameters. Histograms of slowly growing chemostat cultures showed that cell cycle periods B and C + D increase with a decreasing growth rate and that the B period occupies an increasing fraction of the cycle. The DNA replication patterns of B/r A and K were found to be quite similar. At extremely low growth rates (doubling time [T] = 17 h), B/r A cells had a B period of 0.8 T, a C period of 0.1 T, and a D period of 0.1 T, and B/r K cells (T = 16 h) had a B period of 0.6 T, a C period of 0.15 T, and a D period of 0.25 T. Mass increase, i.e., essentially protein synthesis, was seen in all three periods of the cell cycle. For B/r A cells, the average rate of mass increase was 11 times greater in the D period than in the B period, whereas for B/r K cells the rate of mass increase was twice as great in the D period as in the B period. The DNA and cell size distributions of batch cultures in exponential growth were found to vary with time, indicating that such cultures are not suitable for studies of cell cycle kinetics.

General agreement exists about the timing of chromosome replication in rapidly growing cultures of *Escherichia coli* (3, 12). For slowly growing cultures, however, a variety of patterns of DNA synthesis have been observed. These patterns can be described by four parameters; B, time between cell division and the initiation of a new round of chromosome replication; C, time between initiation and the termination of a round of chromosome replication; D, time between the termination of chromosome replication and the next cell division; and T, cell generation time, i.e., time between one division and the next (mean value for the culture).

At growth rates less than 1 doubling per h, the C period is not constant (2, 3, 8, 13, 14) but increases with decreasing growth rate. Experiments with various techniques and different substrains of *E. coli* B/r have given significantly different values for the relative durations of B, C, and D. Evidence for different replication patterns in the substrains was presented by Helmstetter and Pierucci (8), using the membrane elution technique, and by Koppes et al. (10), using electron microscopic and autoradiographic analyses. Both groups observed one replication pattern with strain B/r A (no B period, long D period) and another pattern with strains B/r K and B/r F (both an initial B and a terminal D period). The results obtained by Kubitschek and Newman (13), who studied cell survival after the radioactive decay of ^{125}I incorporated into the DNA, do not show significantly different patterns for strains B/r A, K, and F.

We studied the growth of *E. coli* B/r A and B/r K cells by dual-parameter flow cytometry. With this technique, measurements of the cellular contents of DNA and protein in single cells can be made with a precision of a few percent and at a rate of 10^4 cells per s. Flow cytometry has proven extremely useful for studies of cell cycle kinetics in eucaryotic cells and its usefulness is now also being established for procaryotic cells (18–20; E. Boye, H. B. Steen, and K. Skarstad, J. Gen. Microbiol., in press). The dual-parameter mode permits a simultaneous view of DNA and protein synthesis. By recording information on single cells in large numbers, this method makes it possible to distinguish subpopulations of cells and thereby resolve details that may not be detected by other methods.

In the present study, dual-parameter histograms were used to determine the length of the B, C, and D periods of *E. coli* B/r A and B/r K and to study the kinetics of protein synthesis in these substrains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Two strains of *E. coli* were used: B/r A and B/r K (from H. E. Kubitschek). Both strains had been designated B/r until differentiation into substrains A and K (8).

Batch cultures were grown slowly at 37°C with bubbling aeration in M9 salts (1) supplemented with 2 mg of L-proline or sodium succinate per ml and rapidly in K plus glucose medium (M9 salts, 1% Casamino Acids, and 11 mg of glucose per ml). Culture doubling times were established from cell counts (viable titer on agar plates).

Steady-state chemostat cultures were limited with 100 μg of glucose per ml in M9 salts at 37°C. The cell doubling times were determined from the values of the culture volume doubling times (7).

Fixation and staining. Bacterial culture samples were fixed by 10-fold dilution in ice-cold 70% aqueous ethanol and stored at 4°C overnight. The fixed cells were washed twice in 0.1 M Tris–0.6% NaCl (pH 7.4) and then suspended in 0.1 M Tris–0.6% NaCl–25 mM MgCl containing the DNA-specific fluorescent dyes mitramycin (Pfizer Inc., New York, N.Y.; 50μg/ml) and ethidium bromide (Calbiochem, La Jolla, Calif.; 25 μg/ml).

Flow cytometry. Flow cytometric measurements were performed with a laboratory-built microscope-based flow cytometer described in detail elsewhere (17, 21). In this instrument, the stained cells, carried by a laminar flow of water, pass one by one through the focus of a fluorescence microscope so that each cell gives rise to a pulse of fluorescence light, the intensity of which is proportional to the cellular DNA content. A sensitive photomultiplier tube transforms the fluorescence pulses into equivalent electrical pulses, which are sized and stored by a multichannel pulse-height analyzer (MCA). The amount of excitation light scattered by each bacterium is detected through a secondary microscope in a dark-field configuration. The resulting pulses are stored by the same MCA. The 63 by 63-channel MCA thus accumulates a three-dimensional histogram of the bacteria with regard to scattered light and their contents of DNA-associated fluorescent dye. DNA-associated fluorescence is a true measure of the DNA content per cell, and the amount of scattered light from an individual bacterium is proportional to the cellular dry mass, i.e., essentially protein content. (E. Boye et al., in press). Fluorescent latex particles (diameter, 0.57 μm; Polysciences Inc.) were added to the samples to monitor the sensitivity and stability of the instrument during sample measurements.

RESULTS.

Calibration of the fluorescence axis. To calibrate the fluorescence axis with regard to cellular DNA content, we measured the fluorescence from bacteria grown in the presence of chloramphenicol. Chloramphenicol is an inhibitor of protein synthesis enabling the replication forks to finish rounds of replication while blocking the initiation of new rounds of replication and cell division. Cells grown in the presence of chloramphenicol therefore end up with an integer number of fully replicated chromosomes (18, 19). A sample of chloramphenicol-treated cells was used as a fluorescence standard in all experiments, giving a good measure of the fluorescence per chromosome. The DNA staining depended to some degree on the growth conditions, conceivably reflecting differences in chromosome configuation.

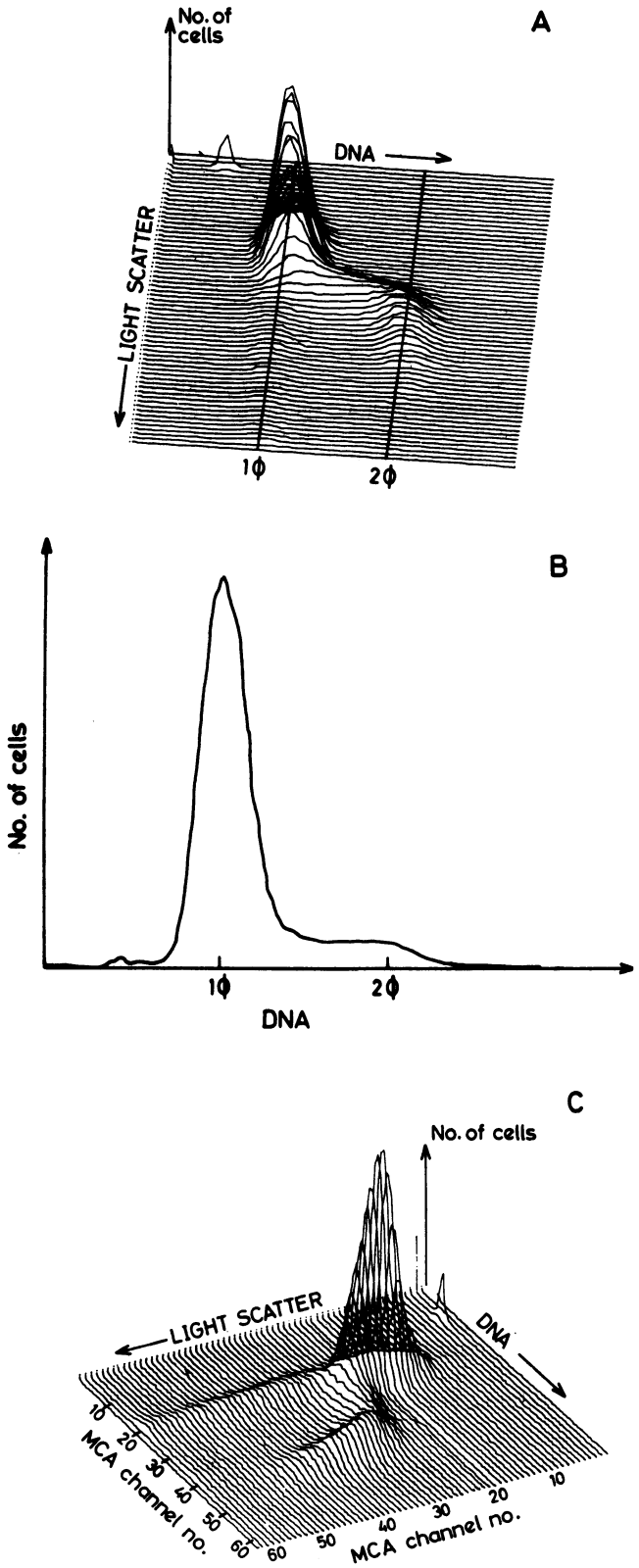
Steady-state chemostat cultures. In a slowly growing chemostat culture (T = 17 h) of strain B/r A, most of the cells had a DNA content equivalent to one single chromosome (Fig. 1A). These cells were in the B period of growth. Cells containing two full chromosomes were in the D period and had, on the average, twice the mass and protein contents of the cells with one chromosome. The ridge of cells connecting the one- and two-chromosome peaks represents cells in the C period, i.e., the period of DNA synthesis.

To determine the duration of the B, C, and D periods, we integrated the dual-parameter histogram of Fig. 1A with regard to light scatter, yielding a single-parameter DNA histogram (Fig. 1B). From this histogram, the fractions of the cell culture occupying B, C, and D periods were found, and by taking into consideration the exponential multiplication of cells growing in steady-state cultures (22), we made estimates of the cell cycle parameters (Table 1). B occupied approximately 80% of the cell cycle, and C and D occupied approximately 10% each.

Flow cytometric measurements of B/r K cells growing slowly in a chemostat culture (T = 16 h) gave a histogram similar to the one shown in Fig. 1A for B/r A cells (Fig. 2). The cell cycle parameters revealed a similar DNA replication pattern (Table 1), with B, C, and D occupying 60, 15, and 25%, respectively, of the cell cycle.

To get a better view of the size distribution of the cells, we rotated and tilted the three-dimensional histogram of Fig. 1A (Fig. 1C). It can be seen that the ridge of DNA-synthesizing cells is at an angle to the DNA axis indicating that the cell mass increased during the C period. The cells occupying a narrow section of this ridge, for example, DNA channel no. 30 and light scatter channels no. 1 to 63, were at the same stage of replicating a new chromosome. Thus

FIG. 1. Three-dimensional DNA/light scatter histograms of a slowly growing *E. coli* B/r A chemostat culture with a doubling time of 17 h viewed from two different angles (A and C). Each histogram represents 2×10^5 cells, recorded at a rate of about 3×10^3 cells per s with an instrumental resolution of <5%. The three-dimensional histogram was integrated with regard to light scatter, yielding a single-parameter DNA histogram of the slowly growing B/r A cells (B).


A
No. of cells
DNA
LIGHT SCATTER
1φ
2φ
B
No. of cells
1φ
2φ
DNA
C
No. of cells
LIGHT SCATTER
DNA
MCA channel no.
MCA channel no.

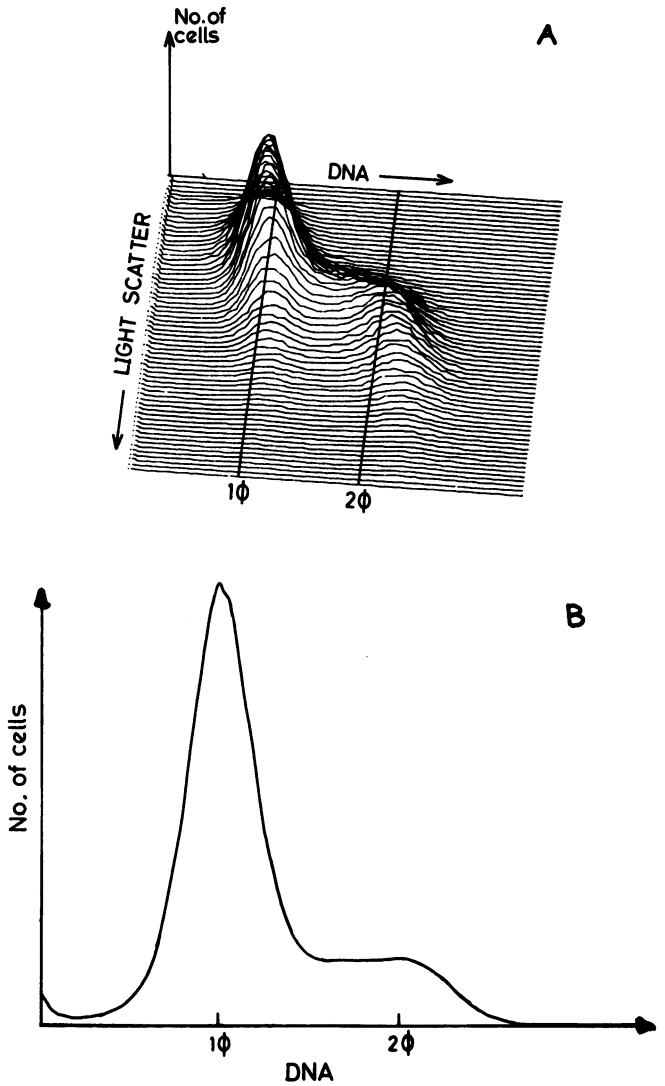


FIG. 2. Three-dimensional DNA/light scatter histogram of a slowly growing *E. coli* B/r K chemostat culture with a doubling time of 16 h (A) and the integrated, single-parameter DNA histogram of this culture (B). In this recording, the light scatter amplifier gain was half of the value used for the histogram of Fig. 1.

these cells were synchronous: their size distribution gave the variability in cell size of the culture at this point in the cell cycle. When the cell size distributions of the DNA channels were considered one by one from the beginning to the end of the C period, the size distributions had about the same width (five channels of the light scatter axis), but moved toward larger light scatter values. This size shift (total of four channels through the C period) indicates the mass increase of the cells in the C period. By taking the duration of the period into consideration, we obtained a measure of the average rate of mass increase in the C period (Table 2). The one- and two-chromosome peaks were considered in the same way, i.e., as resulting from (i) a size variation in cells of the same age (taken to have the same relative width as the size distribution of synchronous cells in the C period) and (ii) an increase in size reflecting a corresponding mass increase. The average rate of mass increase in the D period was about 11 times greater than that in the B period and 4 times greater than that in the C period (Table 2). The size distribution of B/r K cells (Fig. 2A) was somewhat different from that of B/r A cells. Estimates of the rate of mass increase in the three periods gave similar values for the B and C periods and a twofold-higher value for the D period (Table 2).

In Fig. 1C, cells with very large light scatter

TABLE 1. Duration of cell cycle periods for *E. coli* B/r A and B/r K cells

Strain	T	B	C + D	B (min)	C + D (min)	C (min)[a]	D (min)[a]
B/r A	73 min	0.15 T	0.85 T	11	62		
	74 min	0.15 T	0.85 T	11	63		
	95 min	0.25 T	0.75 T	24	71		
	113 min	0.3 T	0.7 T	34	79		
	236 min	0.5 T	0.5 T	118	118		
	293 min	0.5 T	0.5 T	147	147		
	17 h	0.8 T	0.2 T	816	204	102	102
B/r K	231 min	0.4 T	0.6 T	92	139		
	400 min	0.55 T	0.45 T	220	180	60	120
	16 h	0.6 T	0.4 T	576	384	144	240

[a] Separate estimates of C and D are not given when T < 5 h; in these histograms, the two-chromosome peak was not discernible.

signals can be seen as ridges in front of the one- and two-chromosome peaks. The cell cycle distribution of these cells was the same as in the main population of the culture. This fraction of larger cells (about 10%) was not taken into consideration in calculating the rates of mass increase. The nature of these larger cells is not known.

Several other cultures of B/r A and K with doubling times ranging from 73 to 400 min were investigated with dual-parameter flow cytometry. The histograms were reduced to single-parameter histograms as explained above to estimate the cell cycle parameters (Table 1). The B and C + D periods increased with increasing doubling time, and the B period occupied an increasing fraction of the cell cycle. B/r A had a slightly longer B period than did B/r K.

Batch cultures. The DNA and protein contents of batch cultures were found to vary, even during strict exponential growth. In one batch culture of strain B/r A (Fig. 3), exponential growth was maintained for 3.5 h with a doubling time of 78 min (Fig. 3F). Cellular DNA contents can be seen to decrease during the sampling period. Hence, the replication kinetics must have been changing with time. Models for slowly growing cells (T > 1 h) predict a maximum of two replication forks, i.e., one to two chromosome(s) per cell (3), but the cells of Fig. 3A and B have DNA contents as if they were growing with a T < 1 h. Also, cellular protein contents decreased during the sampling period. The average protein content of the cells in Fig. 3A was twice that of the cells in Fig. 3E. This could be expected when the cells approach stationary phase, but there is no indication of this in the growth curve. Other slowly growing batch cultures of B/r A and B/r K cells showed a similar variation in cellular DNA and protein contents during the exponential growth phase. Because of this variability of growth, batch cultures were not used for the determination of cell cycle parameters B, C, and D.

DISCUSSION

The data showed that for B/r A cells, the B period increased with increasing doubling time. C + D also increased, but the fraction of the cell cycle that this period occupied decreased as the doubling time increased. The B/r K cells showed a similar pattern of replication but had a somewhat shorter B period. Our results for slowly growing B/r K cells are in agreement with earlier studies showing a long B period and a short D period (8, 10, 13). Most studies of slowly growing B/r A cells have given cell cycles lacking or having a very short B period (8, 10). This is not in accordance with our results or with the findings of Kubitschek and Newman (13). They found that the C period approached a limiting value of 0.36 T at very long generation times, whereas our measurement gave 0.1 and 0.15 T for the C period of slowly growing B/r A and K cells, respectively. The *E. coli* B/r A used in the present experiments was obtained from H. E. Kubitschek. Since our data agree with those of Kubitschek and Newman, the same flow cytometric experiment was repeated with a B/r A substrain obtained from N. Nanninga. The resulting histogram was quite similar to that of Fig. 2A, giving a B period occupying a major part of the cell cycle.

When growing at a particular rate, rod-shaped bacteria like *E. coli* increase their mass essen-

TABLE 2. Average rate of mass increase in the cell cycle periods of B/r A (T = 17 h) and B/r K (T = 16 h) cells

Strain	Period	Avg rate of mass increase (channels/h)
B/r A	B	0.88
	C	2.4
	D	9.4
B/r K	B	3.1
	C	3.8
	D	6.3

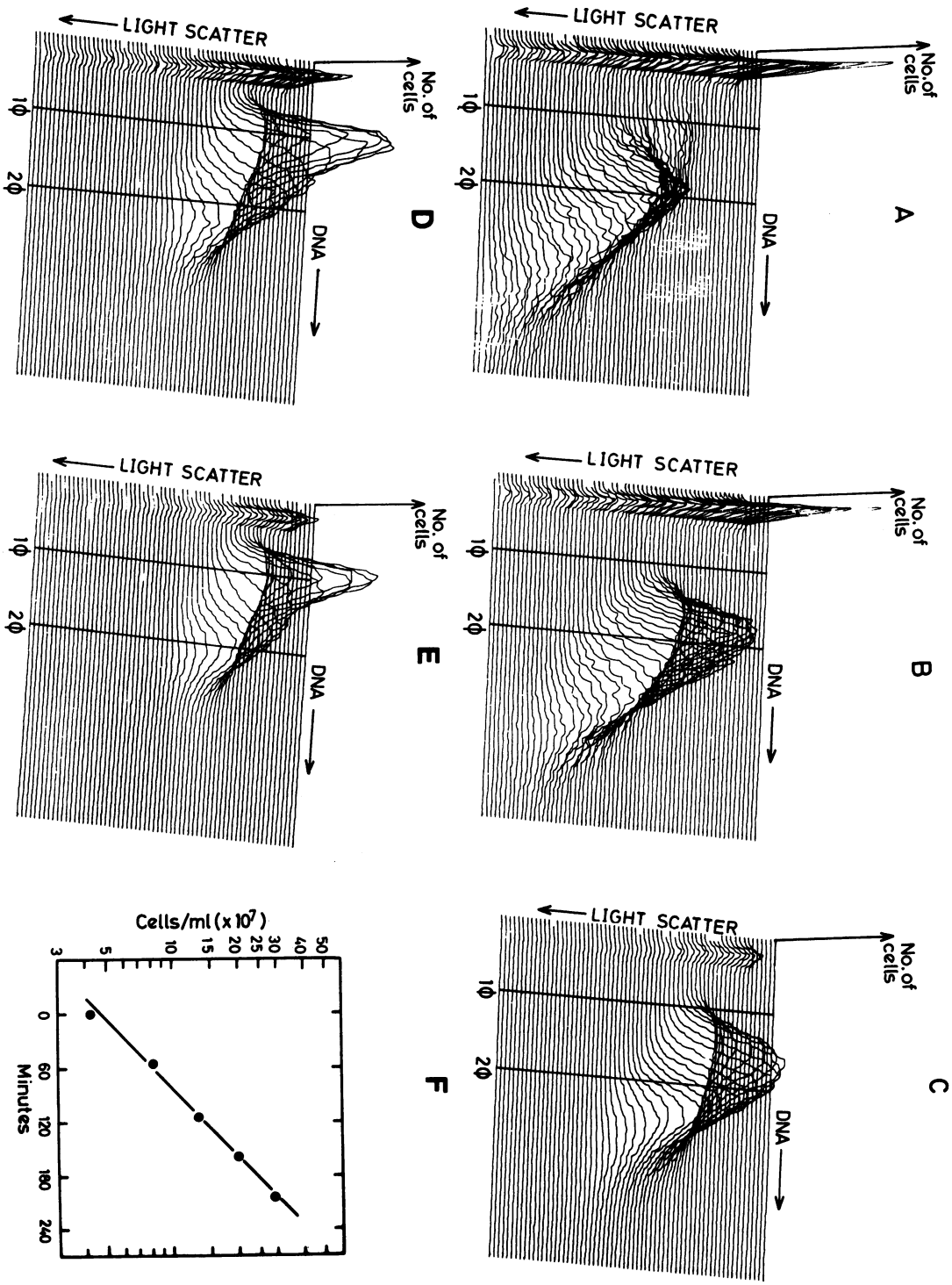


FIG. 3. Histograms of an exponential *E. coli* B/r A batch culture grown in proline medium at 37°C (±0.2°C) (T = 78 min). The samples of (B, C, D, and E) were taken 55, 115, 160, and 250 min, respectively, after the sample of (A). (F) Growth curve for this culture.

tially by elongation: the diameter remains relatively constant (5, 15, 16). Two models of elongation have been presented: exponential (9, 10) and linear (4–6, 11, 16, 23), with a doubling in growth rate somewhere during the cell cycle. Our data on B/r K (T = 16 h) showed a doubling in the average rate of mass increase in the D period compared with the B and C periods. This agrees well with the model of linear elongation, with a doubling in growth rate near the beginning of the D period. The pattern of mass increase for B/r A (T = 17 h), however, was quite different, with a much larger (11 times greater) average rate of mass increase at the end of the cell cycle compared with the beginning. This does not seem to be in accordance with either elongation model. Thus, the two substrains seem to have somewhat different modes of mass increase.

For batch cultures, the distribution of DNA and cell size may change significantly even when the cell culture is growing with a constant doubling time (Fig. 3). This may be one of the reasons why so many different results of the cell cycle parameters of slowly growing cultures have been reported.

Our results demonstrate the efficiency of flow cytometry for obtaining information on bacterial growth. Subpopulations and irregularities in cell cultures are easily and rapidly detected. Thus, the method may also find clinical applications, e.g., in the rapid detection of bacterial sensitivity to drugs.

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