JOURNAL OF BACTERIOLOGY, Apr. 1979, p. 17-23
0021-9193/79/04-0017/07$02.00/0

Vol. 138, No. 1

Length Growth of Two *Escherichia coli* B/r Substrains

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Received for publication 18 January 1979

Length growth of synchronized *Escherichia coli* B/r substrain A (ATCC 12407) and B/r substrain F26 (*thy his*) was followed with an electron microscope. Cells were grown with doubling times (τ) of 60 min (B/r A) and of 82 and 165 min (B/r F26). Different length growth patterns were found for the two substrains. In B/r F, the length growth rate increased about midway in the cell cycle. For τ = 165 min, the rate increase was preceded by a short period of slow growth. For B/r A (τ = 60 min), this period seemed to occur at the beginning of the cell cycle. The possibility is raised that the different length growth patterns are related to different deoxyribonucleic acid replication patterns of the respective strains.

Each rod-shaped *Escherichia coli* cell doubles in size before it divides into two equal daughter cells. Since cell diameter does not change (16) or varies little (unpublished data) during the cell cycle, doubling in size can reasonably be translated as doubling in length. This length doubling can be achieved in numerous ways. Generally, however, workers have tried to distinguish between two alternatives: (i) that length growth is exponential (11, 13, 14) and (ii) that length growth is linear, with a doubling in rate somewhere during the cell cycle (4, 5, 15, 18, 21). Knowledge of the overall length growth pattern is of importance in understanding timing and topography of incorporation of components in the cell envelope (see 13, 20). Eventually one would like to know the exact relationship of length growth to other macromolecular processes inside the cell. Recently, Pierucci (17) presented a model for envelope growth in which new growth zones are activated upon each initiation of DNA replication. We have made a similar suggestion (13).

The analysis of length growth in rod-shaped bacteria has been pursued in a number of ways. In the pioneering studies of Collins and Richmond (3) and of Schaechter et al. (19), length increase of individual cells in a microculture was followed with a light microscope. Later, synchronously growing cells were used to measure (i) mean cell volume electronically with a Coulter Counter (15, 21) and (ii) total cell length with a light microscope (4). Another approach has been to determine the growth kinetics of individual cells from the shape of size distributions of an exponential culture growing in a steady state (3, 6, 12-14).

The aforementioned studies have not led to general agreement with respect to overall length growth of rod-shaped bacterial cells. The reported differences have been caused, at least in part, because of limitations of the techniques employed. When length is measured with a light microscope (4, 19), problems may be encountered because of the small size of bacterial cells. Such problems are perhaps better overcome by using electronically measured volume distributions (15).

In this paper we present electron microscopic length measurements of synchronously growing *E. coli* B/r substrain F26 and B/r substrain A (ATCC 12407). These substrains differ in location of the DNA replication period within the cell cycle (9, 14). In B/r F26, DNA replication starts sometime after cell birth, whereas in B/r A the onset of DNA replication more or less coincides with cell birth. We expected that the resolving power of an electron microscope would enable us to reveal more fine details about the overall cellular growth pattern than previously used instruments did (light microscope and Coulter Counter). In both strains a change of growth rate during the cell cycle was observed. In B/r F26, this occurred about halfway through the cell cycle. In B/r A, a decreased growth rate was seen just after birth. The length growth pattern seems to be substrain dependent and appears more complex than simply linear length growth with a doubling in rate or exponential length growth.

MATERIALS AND METHODS

Culture and medium. *E. coli* B/r A (ATCC 12407) and B/r F26 (*thy his*) were obtained from C. E. Helmstetter. Cells were grown in the minimum salts medium of Helmstetter (7) supplemented with L-alanine

(0.04%) and L-proline (0.04%) (B/r A: doubling time [τ] = 60 to 70 min; B/r F26: τ = 80 to 85 min) or with sodium acetate (0.2%), thymine (20 μg/ml), and histidine (80 μg/ml) (B/r F26: τ = 165 min). Growth was measured turbidimetrically at 450 nm, and cell number was determined with a Coulter Counter (model Z_B) with a 30-μm orifice.

Synchronization. Synchronization was carried out by the membrane elution technique of Helmstetter (8). A culture (100 ml) which was grown exponentially for at least eight generations was poured on a membrane filter (diameter, 142 mm; pore size, 0.22 μm; Millipore Corp., Bedford Mass.) at an optical density of 0.4 and washed three times with prewarmed medium. Then the filter was turned upside down, and elution with fresh prewarmed medium was carried out for 1 h at a rate of 10 to 12 ml/min. In the case of slow-growing cells (B/r F26), better results were obtained when the elution rate was reduced twofold just before newborn cells were collected. After 1 h of preelution (see above), newborn cells were collected in an ice bath (10) for about 1/10 of the doubling time so that a sufficient number was obtained to initiate synchronous growth.

Agar filtration. The modified agar filtration procedure of Woldringh et al. (22) was used. Spreading of cells was promoted with 1% bacitracin instead of sodium dodecyl sulfate (22). Cell length was measured from electron micrographs projected onto a transparent screen at a final magnification of ×10,000 or ×12,000.

RESULTS

We chose to use *E. coli* B/r substrain F26 and B/r substrain A because they can be synchronized by the membrane elution technique (9). In addition, DNA replication cycles under different growth conditions are known for these strains (9, 14).

Length growth of *E. coli* B/r F26. Synchronized cultures were used with doubling times of 82 and 165 min. Cell number was followed with a Coulter Counter or with an electron microscope. Length distributions were made for the exponentially growing cultures (before membrane elution) and at successive times during synchronous growth. These data are shown in Fig. 1 for B/r F26 growing with τ = 82 min. Note that the first frame in Fig. 1 refers to the length distribution of exponentially growing cells. In the length distributions, the constricted cells are indicated by the hatched areas. One observes clearly the emergence and disappearance of the constricting cells. Parameters of the length distributions are listed in Table 1. The change of total cell length (4) with time is shown in Fig. 2. Total cell length represents the relative cell number times the arithmetic mean of the cell length at the given times. A striking dip occurred about midway in the cell cycle, followed by an increase in growth rate at about t (time) = 50 min. This phenomenon was likewise visible during the second generation at about t = 130 min. Immediately after division, the length growth rate was reduced again (roughly fourfold). A broken line has been drawn to indicate exponen-

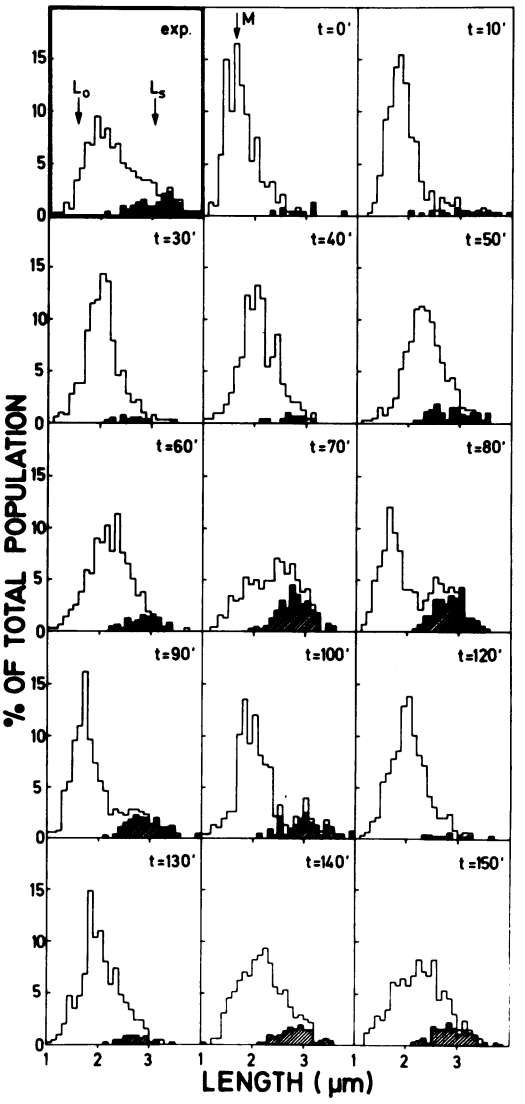


FIG. 1. *E. coli B/r substrain F26 (τ = 82 min) length distributions in an exponential culture (exp.) and at various times during synchronous growth. Constricting cells are indicated by the hatched areas. Each length distribution is composed of about 450 cells. L_0, Average length of the newly born cell; L_s, average length of the separating cell. L_0 and L_s were determined by the method of Harvey et al. (6) from the length distribution of the exponential culture. M, Modal cell length at time zero.*

TABLE 1. *Parameters of length distributions of E. coli B/r F26 (τ = 82 min)*

Time (min)	Mean length (mm)[a]	Coefficient of variation (%)	Skewness	Kurtosis	Dividing cells (%)
0	17.5	22.0	1.57	3.68	4.4
10	19.0	22.7	1.79	3.89	5.8
30	20.0	17.2	0.46	0.90	3.0
40	20.4	17.9	0.29	0.20	3.7
50	22.9	17.6	0.26	0.33	12.3
60	21.7	19.6	0.17	0.03	9.4
70	24.3	22.5	−0.17	−0.69	28.0
80	20.4	27.9	0.49	−0.96	25.7
90	19.5	27.2	1.08	0.48	17.1
100	21.3	25.0	1.11	1.05	16.5
120	20.2	18.8	0.61	0.76	2.9
130	20.1	20.0	0.40	0.05	5.4
140	21.6	21.5	0.22	−0.32	14.4
150	22.5	22.5	0.24	−0.31	17.4

[a] 1 mm = 0.1 μm.

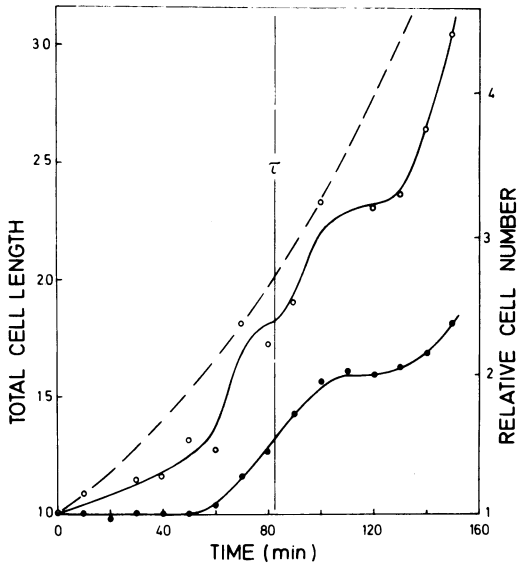


FIG. 2. *Total cell length as a function of time in synchronized E. coli B/r substrain F26 (τ = 82 min). ○, Relationship between length and time of the first and second generation; ●, relative cell number as determined with a Coulter Counter. The broken line denotes exponential length growth.*

tial growth of length. The exponential curve has been drawn from the total cell length at $t = 0$ to twice the total cell length. If the curve had been drawn from total cell length at t = 82 min to half this total cell length, the exponential curve would have been nearer the experimental points. However, this does not affect our main conclusions. The same reasoning applies to Fig. 5 (see below). It can be seen that the length growth pattern deviated from exponential growth and from linear growth with a doubling in rate. The mean cell length at $t = 0$ (1.75 μm; Table 1) and the modal cell length at $t = 0$ (1.60 μm; Fig. 1) were somewhat larger than the average cell length (1.55 μm; Fig. 1) of the newborn cell. The larger mean cell length may have been caused by contaminating older cells. We also plotted modal cell length against time (not shown). In this case, the same overall length growth pattern was found.

To measure the degree of synchronization, we calculated the synchronization index (F) by the method of Blumenthal and Zahler (2). We found that $F = 0.32$. To appreciate this value, consider a synchronous culture with τ = 100 min and an age variation at division of 20% (19). In this case, $F = 0.27$.

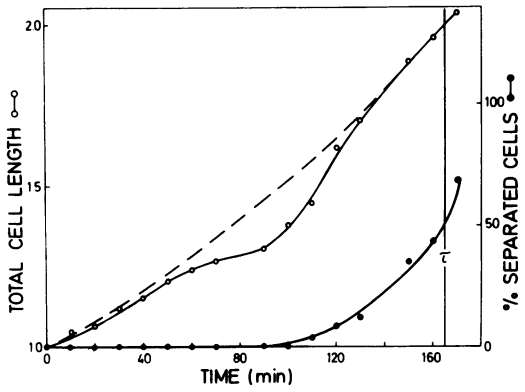
We also studied the length growth pattern of B/r F26 growing synchronously ($F = 0.22$) with τ = 165 min (Table 2). A similar picture was obtained (Fig. 3): a slowing down of elongation about halfway through the cell cycle. The broken line in Fig. 3 represents exponential length growth. (The doubling time of the exponential culture applied to the elution filter was also 165 min.) These results thus indicate that length growth in B/r F26 neither is simply linear with a doubling in rate nor is exponential.

Length growth of *E. coli* B/r A. *E. coli* B/r A is one of the most intensively studied strains in cell cycle research. A basic difference between B/r A and B/r F26 is the location of the DNA replication period within the division cycle. In

TABLE 2. *Parameters of length distribution of E. coli B/r F26 (τ = 165 min)*

Time (min)	Mean length (mm)[a]	Coefficient of variation (%)	Skewness	Kurtosis	Dividing cells (%)
0	14.0	24.7	1.51	2.30	3.8
10	14.3	23.8	1.51	2.62	3.9
20	14.6	22.2	1.53	2.48	3.9
30	15.3	21.4	1.44	1.97	4.6
40	15.6	21.1	2.25	8.89	4.5
50	16.1	18.7	1.37	2.82	5.1
60	16.6	19.8	1.37	2.79	4.4
70	16.6	18.4	1.21	2.39	4.5
80	16.9	17.3	0.49	0.39	5.6
100	17.9	18.6	0.25	0.15	7.9
110	17.9	18.5	−0.02	−0.36	11.2
120	19.1	19.6	−0.15	−0.49	19.2
130	19.5	20.9	−0.23	−0.65	25.9
140	18.4	23.0	−0.14	−0.93	25.6
150	18.2	27.05	0.10	−1.17	29.7
160	17.8	27.9	0.24	−1.01	24.5
170	15.8	28.9	0.86	−0.20	17.1

[a] 1 mm = 0.1 μm.



B/r A, DNA replication more or less coincides with the birth of daughter cells, whereas in B/r F26 it takes some time before replication starts. These differences are most apparent in slowly growing cells (9, 14; unpublished data). Figure 4 represents length distributions of an exponentially growing culture (τ = 60 min) and of samples taken at successive times from the synchro-

FIG. 3. *Total cell length as a function of time for synchronized E. coli B/r substrain F26 (τ = 165 min). Symbols as in Fig. 2. Cell number was determined by electron microscopy from the corrected percentage of separating cells.*

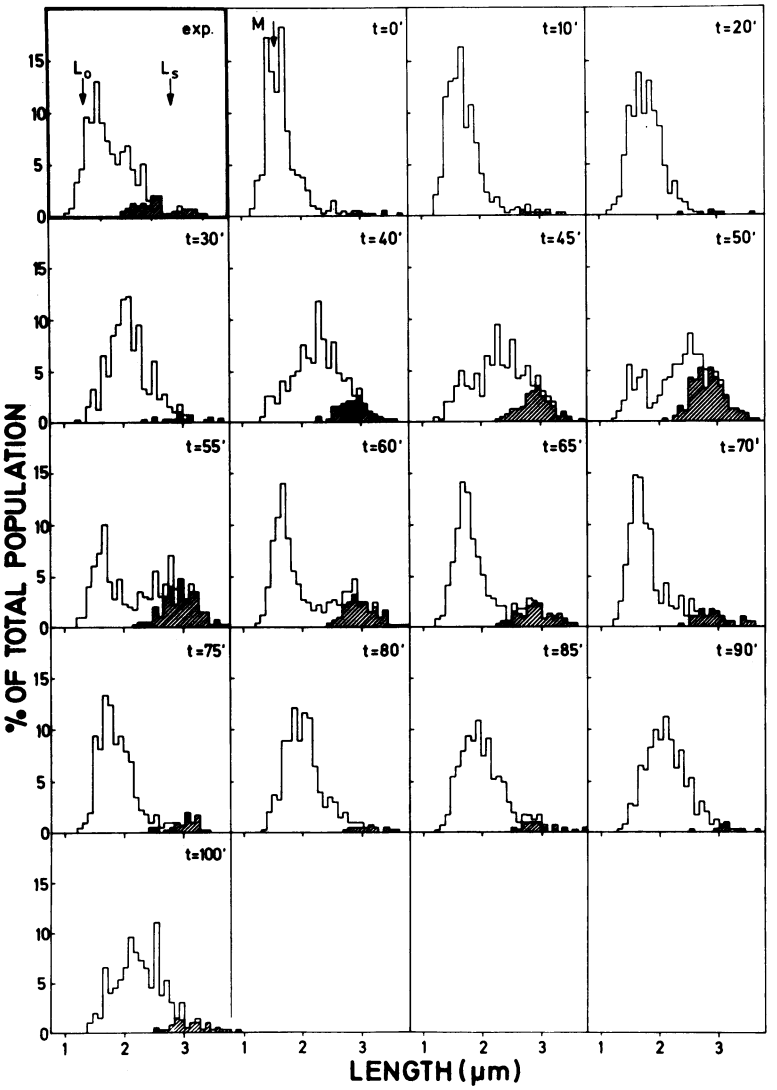


FIG. 4. *E. coli B/r substrain A (τ = 60 min) length distributions. Description as for Fig. 1. Each length distribution is composed of about 600 cells. Note that the scale of the exponential culture was slightly shifted to the left. For L_0 and L_s see the legend to Fig. 1. M, Modal cell length at time zero.*

nized culture (τ = 60 min; F = 0.33). Parameters of the length distributions are listed in Table 3. The same presentation has been adopted as for B/r F26 (Fig. 1). Thus, total length is plotted against time in Fig. 5. A slowing down of length growth seemed to occur at the beginning of the cell cycle. This growth pattern was again visible in the second generation. The pattern clearly differs from that of B/r F26 (Fig. 2 and 3). Again the interrupted line in Fig. 5 represents exponential cell elongation.

We considered whether or not the comparatively slow growth at the beginning of the division cycle of B/r A is an artifact. One might argue that the phenomenon occurs because cells were collected in the cold before the start of synchronous growth or that cells were somehow impaired after leaving the membrane filter. Several arguments suggest that this is not the case: (i) exponentially growing *E. coli* B/r A cells stored in the cold for as long as 100 to 200 min resumed growth without any observable delay and with the same doubling time as was measured by optical density (data not shown); (ii) the doubling time of the exponentially growing culture which was applied to the filter was about the same (65 min) as that of the synchronous culture (60 min); (iii) when synchronous growth was followed beyond the first generation, a slowing down of growth was again observed at the emergence of the second generation (Fig. 5); (iv) slow growth as such may take place during the cell cycle, as was most clearly shown by slowly growing B/r F26 (Fig. 3).

TABLE 3. *Parameters of length distributions of E. coli B/r A (τ = 60 min)*

Time (min)	Mean length (mm)[a]	Coefficient of variation (%)	Skewness	Kurtosis	Dividing cells (%)
0	20.0	19.6	2.18	6.84	1.8
10	20.6	18.6	1.84	4.75	1.9
20	21.5	16.3	1.17	3.36	1.4
30	25.4	18.2	0.99	1.94	4.6
40	27.1	19.0	0.20	−0.08	17.5
45	27.8	20.7	0.07	−0.51	22.8
50	28.3	21.8	−0.18	−0.71	40.5
55	27.1	26.9	0.12	−1.27	23.7
60	25.0	27.7	0.72	−0.80	22.2
65	23.9	25.8	1.27	1.07	15.7
70	22.5	24.4	1.42	1.46	11.3
75	23.1	21.5	1.40	1.61	7.7
80	24.3	18.2	1.15	1.69	3.9
85	24.2	20.5	1.14	1.67	7.5
90	25.4	17.2	0.67	0.54	2.3
100	27.2	19.3	0.64	0.91	6.6

[a] 1 mm = 0.1 μm.

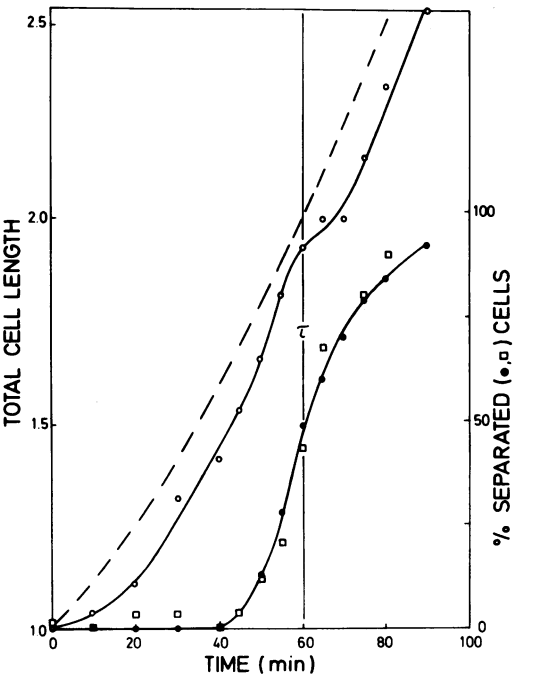


FIG. 5. *Total cell length as a function of time in synchronized E. coli B/r substrain A (τ = 60 min). Symbols as in Fig. 2. Cell number was determined with a Coulter Counter (□) and with an electron microscope from the corrected percentage of separating cells (●).*

DISCUSSION

Our data indicate that overall length growth of synchronized populations of *E. coli* B/r substrain F26 (τ = 82 min; τ = 165 min) and B/r substrain A (ATCC 12407; τ = 60 min) is rather complex. That we found a complex pattern is not so surprising since so many elements are involved in cellular growth. The complexity is apparent when the two bacterial substrains are compared. In B/r A, a period of slow growth seemed to occur at the beginning of the division cycle. This was followed by accelerated length growth. In B/r F26 (τ = 165 min), this occurred about halfway through the cycle, whereas for τ = 82 min length growth increased considerably at about τ = 50 min.

It is relevant to consider that the membrane elution technique may affect cell growth kinetics. The ideal situation would be one in which the growth of single cells in a culture chamber is followed with the accuracy of an electron microscope. Such a situation does not yet exist. A point of concern in our case could be the fact that we collected newly born cells in the cold before the start of synchronous growth. Slow growth at the beginning of the cell cycle of B/r

A (Fig. 5) may have been the result of a traumatic experience of the bacterial cells. In Results we have presented arguments which led us to believe that cold shock did not affect cell growth in a measurable way.

Another concern is the quality of synchronization. As a measure of this we calculated synchronization indexes (see Results) and compared our results with those of others. Unfortunately, this is not a common practice, but we calculated F values from a number of published synchronization curves. It appears that our synchronization results are quite reasonable. One should also consider that variation in the age of division can amount to 23% (19) and that a variation is by necessity present in each synchronization by membrane elution.

Comparison with other data. Direct observation of *Bacillus cereus* (3), *Salmonella typhimurium*, and *E. coli* B/r (19) cell lengths in culture chambers led to the idea that length growth is exponential. We cannot confirm these results with our approach. It is possible that our synchronization procedure disturbed the normal growth cycle (in an unknown way) or, alternatively, that the resolving power of the light microscope represents a limiting factor. Cell size (volume or length) has also been followed in synchronized populations. Kubitschek (15) measured with a Coulter Counter cell volumes of synchronously growing *E. coli* cells which had been selected by centrifugation. He found linear growth, with a doubling in growth rate at division. Ward and Glaser (21) synchronized an *E. coli* B/r strain with the membrane elution technique. Their measurements suggested linear volume growth, and the time at which the rate of volume growth doubled was found to coincide with the start of new rounds of DNA replication. Donachie et al. (4) isolated small cells of *E. coli* B/r A (ATCC 12407) by centrifugation to start synchronization; cell lengths were measured with a light microscope. Their data points (Fig. 3b in reference 4) for *E. coli* B/r A are not incompatible with ours (Fig. 5). However, we cannot support their general conclusions, based on the calculation of total cell lengths, that length growth is linear and that the rate doubles (at a size of twice the minimum cell length) 20 min before division.

The above-mentioned disagreement is due in part to methodological differences. We feel, however, that the superior resolving power of an electron microscope is a considerable advantage. Admittedly, we are looking at dehydrated specimens, and the underlying assumption is that the drying process affects cells in all length classes in the same way.

Cell growth and DNA replication. An intriguing question concerns the cause of the different length growth patterns of B/r substrains A and F26. The two strains differ in timing of the DNA replication cycle during slow growth (9, 14; unpublished data). It is therefore tempting to relate the overall elongation pattern to DNA replication. In the two strains the period of change of length growth rate seems to occur around (after) initiation of DNA replication, i.e., in B/r A at the beginning of the cell cycle and in B/r F26 further on. In the experiment with B/r F26 (τ = 165 min), we found by autoradiography (13 and 14) that initiation of DNA replication took place at t = 61 min (manuscript in preparation). Ward and Glaser (21) observed a change in the linear volume increase of *E. coli* B/r at the initiation of DNA replication, although they did not find a tight coupling between the two processes. In our case, we cannot be definite about the importance of initiation of DNA replication with respect to cell elongation; however, an alteration of the growth pattern near termination of DNA replication (5, 23) does not seem to apply (1, 13) because no change in the cell elongation pattern could be seen towards the end of the cell cycle (Fig. 2, 3, and 5).

Age and length in an exponential culture. Some authors have tried to deduce the growth of individual cells from the shape of the size (volume or length) distributions of an exponential culture. This can be done with the formula developed by Collins and Richmond (3) or the modifications thereof by Koch (11). Assessments of the size distributions of the newborn and separating cells must be made. Application of the Collins-Richmond formula (3) to *E. coli* PAT84 (13) and to *E. coli* B/r A and B/r K (14) revealed an exponential relationship between length and relative age. Since the present data do not support such a relationship, we carried out a Collins-Richmond analysis for *E. coli* B/r F26 (τ = 80 min; τ = 165 min) and for B/r A (τ = 60 min). In no case could an exponential mode of elongation of individual cells be excluded (L. J. H. Koppes et al., unpublished data).

The question thus arises as to which approach is the best. In the size distribution approach, cells are arranged according to length, irrespective of age. A relative cell age per length class can be estimated, and even the theoretical length distributions of extant cells can be reliably obtained (13, 14; unpublished data). However, we feel that a better approach is to use age as the primary parameter and to measure cell length as it changes with time. This approach is the most straightforward one. As mentioned above, the dilemma will be resolved as soon as

the length growth of large numbers of individual cells can be followed with the same precision as that with an electron microscope.

ACKNOWLEDGMENTS

We thank C. L. Woldringh for discussions and L. J. H. Koppes for valuable help with the calculations. We also thank J. D. Leutscher for preparing the diagrams.

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