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Effect of Thymine Concentration on Cell Shape in Thy⁻ *Escherichia coli* B/r

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Cells of a thymineless mutant of *Escherichia coli* B/r are shown to change their shape when the concentration of thymine in the growth medium is reduced. Electron micrographs of whole cells and isolated sacculi were used to make quantitative measurements of the changes in cell length and width which occur as a result of such a change in thymine concentration. The results showed that there is an increase in cell volume, which is due to an increase in cell width accompanied by a decrease in cell length. These changes were compared with the predictions of models which assume that cell shape is influenced by the chromosome replication cycle.

Cells of *Escherichia coli* and related bacteria extend only in length during steady states of exponential growth (15), but the increase in cell size associated with either an increase in growth rate (9, 23) or a reduction in the rate of deoxynucleotide addition at a replication fork (the "replication velocity") (21, 28) is accompanied by an increase in cell diameter. This increase in diameter, which must take place only during the transition from one steady state to another, may provide important information about the determination of cell shape and the timing and mechanism of cell division (18, 19). It therefore seems desirable that an accurate quantitation of the changes in cell shape under different growth conditions be obtained. The present paper is concerned with the change in shape associated with a reduction in replication velocity in a thymineless strain of *E. coli* B/r.

On the basis of preliminary measurements it was concluded (28) that the major change in cell shape associated with a decrease in replication velocity (an increase in the replication time, *C*) in an *E. coli* 15T⁻ strain was an increase in cell diameter. Average cell length appeared to be reduced. These conclusions were based on the measurement of widths of whole cells fixed and air dried on electron microscope grids. The change in average length was not measured directly but was estimated from the measured diameters and average cell volume, which was assumed to be equivalent to cell mass and was determined as absorbance per cell. A potential source of error in such measurements is that cells might flatten during drying and the degree of flattening might differ in cells grown under different conditions. In the present paper this difficulty has been overcome by isolating sacculi (24), which we show to flatten completely during drying. Widths of sacculi isolated from cultures of an *E. coli* B/r Thy⁻ strain growing in the presence of different thymine concentrations, and therefore having different replication times, were compared. In addition, the length distributions of both whole cells and sacculi from the same cultures were measured directly.

The data confirm the earlier conclusion that the increase in cell size associated with an increased replication time is due to an increase in cell diameter and a reduction in average cell length.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. The bacterium used for these experiments was the *E. coli* B/r Thy⁻ (*tlr*) strain LEB16 described previously (16). Growth medium consisted of minimal salts solution (8) supplemented with 0.2% (wt/vol) glucose as carbon source and thymine at the concentrations indicated in each experiment. Cultures were incubated with vigorous aeration at 37°C in a New Brunswick gyratory shaker. Measurements of culture mass, particle number, and relative DNA content were made when the culture had attained a steady state of exponential growth (3) as described previously (16, 21).

Isolation of murein sacculi. Sacculi were prepared by the method of Ryter et al. (22). Cells from 40-ml samples were pelleted and resuspended in 2 ml of ice-cold water and added slowly to a 20-ml boiling aqueous solution of 4% sodium dodecyl sulfate. After boiling for 5 min, the solution was cooled and centrifuged for 60 min (39,000 × *g*) at 20°C. The pelleted sacculi were washed by resuspension and recentrifugation and finally resuspended in 0.04% sodium dodecyl sulfate solution.

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Whole-cell preparations. Cells to be examined in the electron microscope were fixed with osmium tetraoxide by Kellenberger's standard fixation procedure (14). Samples (40 ml) of culture were added to 4 ml of Kellenberger's fixative (14) and centrifuged immediately for 5 min (3,000 × *g*); the pellet was resuspended in 1.0 ml of Kellenberger's fixative and 0.1 ml of tryptone medium and allowed to stand overnight at room temperature. The suspension was diluted with 8 ml of Kellenberger's buffer (14) and centrifuged for 5 min (18,000 × *g*). Finally, the fixed cells were washed and resuspended in distilled water.

Electron microscopy. Samples of murein sacculi and fixed cells were mounted onto collodion films by the agar filtration method (13). All samples examined included polystyrene latex beads (Polaron M184-2) of known diameter (0.308 μm) as a calibration standard (12). Mounted sacculi preparations were shadowed with platinum to increase their definition. Cell dimensions were obtained from electron micrographs projected to a final magnification of about 45,000. The number of cells measured in each experiment is recorded with the relevant results.

RESULTS

Dimensional changes of whole cells and isolated sacculi. Previous electron microscopic measurements (26, 28) of the diameters of bacterial cells growing under different cultural conditions have been unreliable because of uncertainty about the degree of distortion of the cells which may arise during the fixation, mounting, and drying procedures involved. We have attempted to overcome these difficulties of sample compression and shrinkage by using isolated sacculi for width measurements. In addition, we have used the agar filtration technique (13) of sample preparation since this has been shown to cause a minimum of distortion to the specimen under investigation (13; C. L. Woldringh, Ph.D. thesis, University of Amsterdam, Amsterdam, The Netherlands, 1974). Figure 1 is an electron micrograph of a shadowed preparation of a mixture of whole cells, sacculi, and latex spheres. It can be seen that despite the fact that the whole cells have a greater width than the latex spheres, they cast shorter shadows. Clearly there has been significant flattening of the cells during the drying procedure. In addition, the shadow is longer at the cell poles, indicating that these are more resistant to flattening. There is also a less densely shadowed region along the central line of the cells, suggesting that they have collapsed along this line. In contrast, the sacculi cast no detectable shadow, suggesting that they have collapsed completely. They retain the rod shape of the cells from which they were obtained. In our subsequent calculations we have assumed that the circumference of unfixed whole cells is proportional to twice the measured width of the sacculi.

Dimensions of cells cultivated in high and low concentrations of thymine. In Fig. 2 we have plotted the DNA concentration (radioactive thymine incorporated per unit of absorbance) and cell size (absorbance per particle) of a culture of LEB16 growing in glucose minimal medium before, during, and after a transition from medium containing a high concentration (20 μg/ml) to a low concentration (4 μg/ml) of thymine. The growth rate of the culture (doubling time, 40 min) is not affected by this change in thymine concentration. The important features to notice are that before the transition DNA concentration and cell size are both constant, indicating that the culture is in a steady state of exponential growth with respect to these parameters. New steady-state values for the same parameters are attained by 150 min after the step-down to the lower thymine concentration. Extensive evidence has been provided earlier that the change in DNA concentration is due to a reduction in replication velocity in Thy^- cells growing in low thymine concentrations (1, 16, 21, 28). The change in DNA concentration observed in the experiment shown in Fig. 2 is that expected if the replication time has lengthened by 33 min. The observed change in cell size in the same experiment is that predicted if, in addition, the period between completion of rounds of replication and cell division (*D*) has shortened by 9 min (see reference 16 for the method of computing these changes).

Samples for electron microscopic determination of cell lengths and widths were taken immediately before and at 350 min after the step-

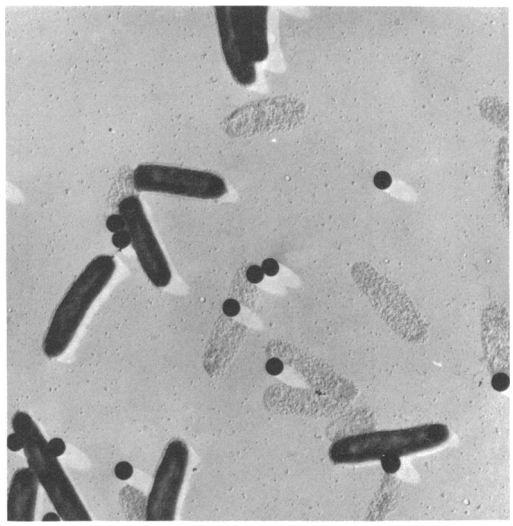

FIG. 1. *Electron micrograph of a shadowed preparation of whole cells, isolated sacculi, and polystyrene latex beads.*

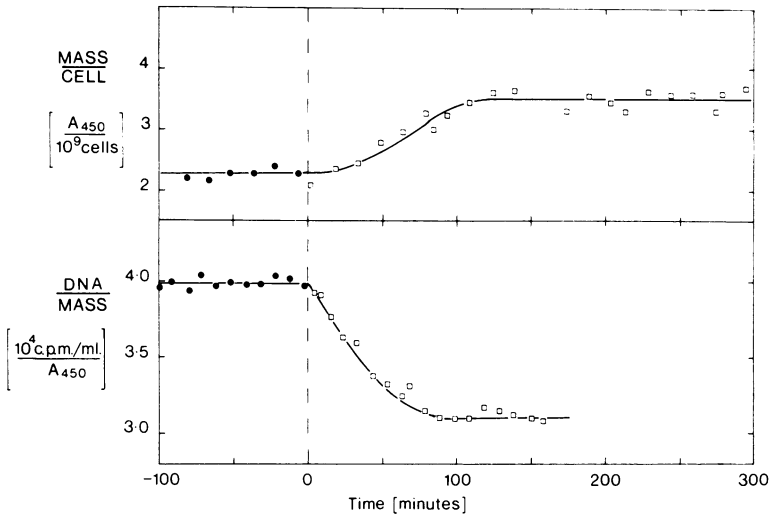

FIG. 2. *Effect of a reduction in thymine concentration on the DNA-mass ratio and average cell size in E. coli B/r Thy⁻. Strain LEB16 was grown in glucose minimal medium containing [^{14}C]thymine (0.05 μCi/μg) and sampled at suitable time intervals for measurement of culture mass (absorbance at 450 nm [A_{450}]) and DNA content (radioactivity in trichloroacetic acid-precipitable material). At 0 min a steady-state exponential culture (A_{450}, 0.3) growing in 20 μg of [^{14}C]thymine per ml (●) was diluted fivefold with prewarmed medium containing no thymine to give a final concentration of 4 μg of thymine per ml (□). The culture was then maintained below A_{450} = 0.3 by dilution with prewarmed medium containing 4 μg of [^{14}C]thymine per ml after each generation of growth.*

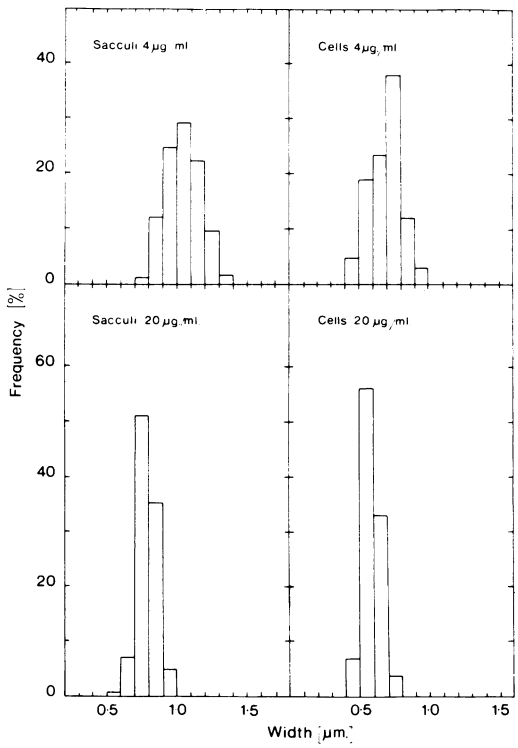

FIG. 3. *Histograms of width measurements taken from electron micrographs of whole cells and sacculi from cultures grown in 20 and 4 μg of thymine per ml.*

down in thymine concentration. Cells from one portion were fixed and mounted while sacculi were prepared from a second portion and then similarly mounted. Width and length distribution were measured for each sample. The data are presented as histograms (Fig. 3 and 4) and numerically (Table 1). Photographs of typical shadowed sacculi and unshadowed fixed cells are shown in Fig. 5.

From the width histograms it is apparent that both cells and sacculi from the 4-μg/ml culture have a greater width than those from the 20-μg/ml culture. The average increase in width is about 19% for cells and 30% for sacculi (Table 1). This suggests that the degree of flattening of whole cells is less in the case of the 4-μg/ml culture than in the case of the 20-μg/ml culture. The width distributions of both cells and sacculi from the 4-μg/ml culture are broader than those from the 20-μg/ml culture. This might indicate that 350 min after the transition from low to high thymine concentrations the culture has still not reached a steady state with respect to this parameter. Longer periods of incubation at the low thymine concentration were not used because previous experiments (16) had shown that after prolonged incubation at this concentration a slow but continuous increase in average cell size takes place.

The average length of whole cells from the 4-μg/ml thymine culture is about 10% less than that from the 20-μg/ml culture (Table 1). In

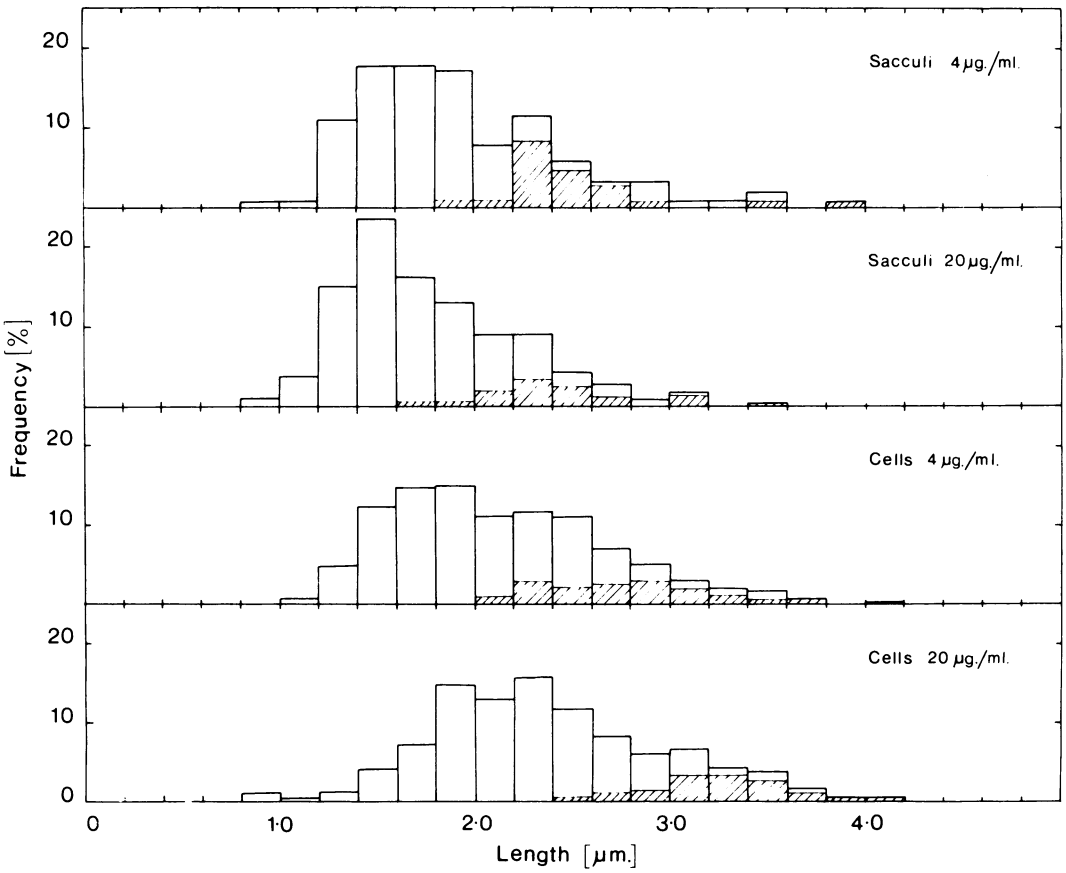

FIG. 4. *Histograms of length measurements taken from electron micrographs of whole cells and sacculi from cultures grown in 20 and 4 μg of thymine per ml. The shaded areas represent cells and sacculi with septa.*

TABLE 1. *Average length and width of cells from cultures growing on different concentrations of thymine*[a]

Thymine (μg/ml)	Cells		Sacculi	
	Width (μm)	Length (μm)	Width (μm)	Length (μm)
20	0.58 ± 0.004 (348)[b]	2.37 ± 0.03 (317)	0.79 ± 0.004 (330)	1.77 ± 0.03 (302)
4	0.69 ± 0.006 (328)	2.13 ± 0.03 (302)	1.03 ± 0.008 (199)	1.92 ± 0.04 (159)

[a] Data are shown ± standard error of the mean.
[b] Number in parentheses is number of cells measured.

both cases the average length of the sacculi is substantially less than that of whole cells. We interpret this as due to the fact that a proportion of cells with partial or complete septa will split in two during the preparation of sacculi. If this interpretation is correct, it would follow that a greater proportion of cells are cleaved in this way from the 20-μg/ml culture than from the 4-μg/ml culture, since the difference in length between sacculi and cells is greater in the former culture (34%) than in the latter (11%). The shape of the length histograms supports this interpretation. The 20-μg/ml sacculi length distribution shows a significant loss of cells from the long-cell classes and an accompanied gain in the small-cell classes when compared with the 20-μg/ml cell length distribution. In the case of the 4-μg/ml culture the changes are similar in nature but appear smaller in magnitude. This is perhaps not unexpected in view of our previous finding (16), which is supported by the present work, that the *D* period is shorter in cultures growing on low concentrations of thymine than it is in cultures growing on high concentrations. Thus

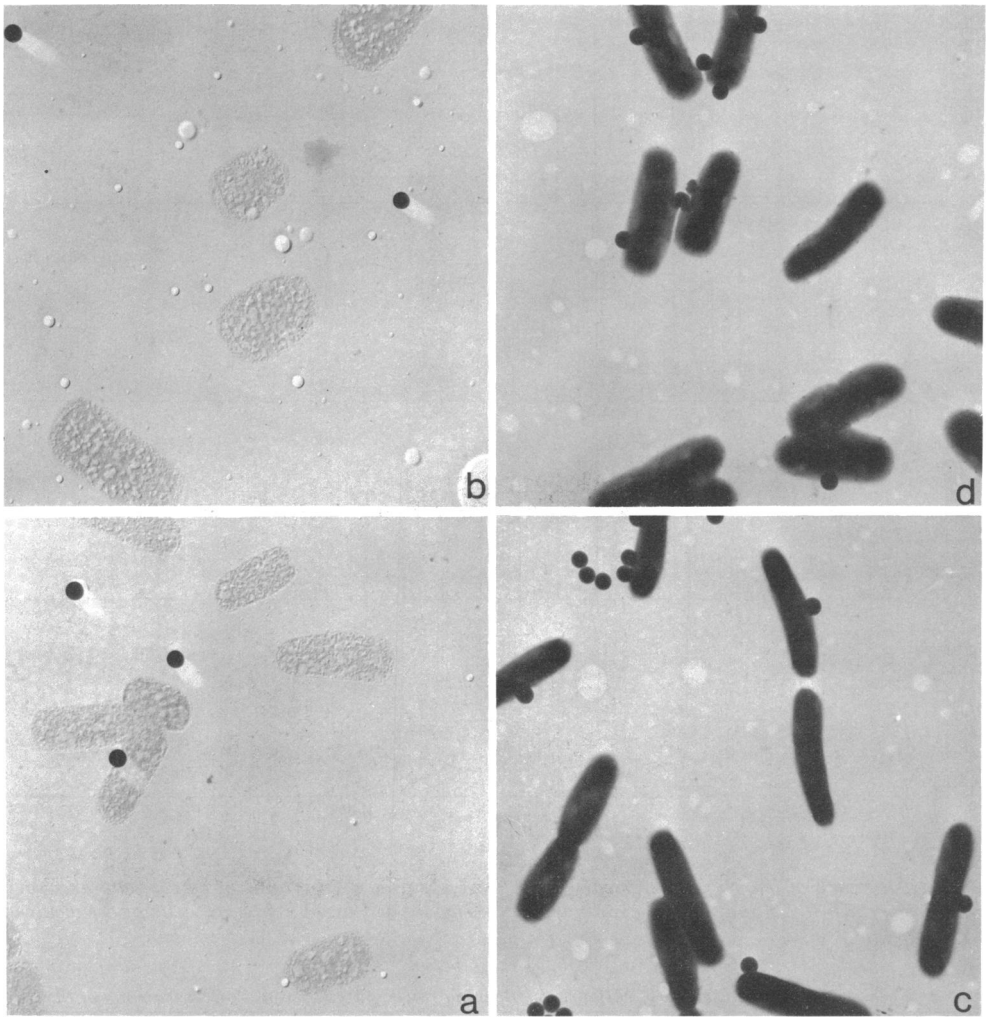

FIG. 5. *Electron micrographs of sacculi and whole cells from cultures growing in different thymine concentrations. Shadowed preparations of sacculi isolated from cultures growing in (a) 20 and (b) 4 μg of thymine per ml. Osmium-fixed cells from cultures growing in (c) 20 and (d) 4 μg of thymine per ml. In all cases the spherical objects are latex beads included as calibration standards.*

the proportion of cells with septa might be less in the former case than in the latter. On the other hand, the proportion of sacculi with visible septa is similar to the proportion of whole cells similarly constricted. It is perhaps possible that not all whole cells with constrictions are identified microscopically. Indeed the percentage of constricted cells from the 20-μg culture (13%) is low in comparison to that found by Woldringh (26) from another strain of *E. coli* B/r growing at a similar rate (23% at a doubling time of 32 min). It is possible that a subjective bias is introduced into the estimation of the proportion of the two cell populations which show constrictions in favor of the low-thymine cells over those grown in high thymine. This is because the increased width and decreased length of the former cells cause them to be more rounded in shape and may thus lead to more pronounced constrictions, which are easier to detect.

Finally, in Table 2 average cell volume is computed from the mean width of sacculi and the mean length of whole cells. We also present the mass-volume ratio obtained from the absorbance per particle measurements from Fig. 2. Since the mass-volume ratio calculated in this way is identical for the 20- and 4-μg/ml cultures, it seems probable that any errors in our width and length measurements are common to both cultures. The table also contains the computed

TABLE 2. *Comparison of cell dimensions obtained from exponential cultures and from direct microscopic observation*

Thymine (μg/ml)	Mass/cell (A_{450}[a]/10^9 cells)	Mean sacculi width (μm)	Cell diam[b] (μm)	Mean cell length (μm)	Cell vol[c] (μm^3)	Surface area[c] (μm^2)	Mass/vol [(A_{450}/10^9 cells)/μm^3]	Surface area/mass [μm^2/(A_{450}/10^9 cells)]
20	2.28	0.79	0.50	2.37	0.43	3.72	5.30	1.63
4	3.44	1.03	0.66	2.13	0.65	4.42	5.29	1.28

[a] A_{450}, Absorbance at 450 nm.
[b] Calculated from sacculi widths.
[c] Calculated assuming cells to be cylinders with hemispherical ends.

surface area and surface area-mass ratio for both cultures.

DISCUSSION

Two questions raised by the observations reported here, and by the similar increase in width which is associated with an increase in growth rate, are why does it take place and how does it take place. A variety of suggestions have been made (18, 19, 27, 28; N. B. Grover, C. L. Woldringh, A. Zaritsky, and R. F. Rosenberger, J. Theor. Biol., in press), all of which have in common the assumption that the rate of synthesis of the cell envelope relative to that of cell mass (and volume) decreases as the growth rate increases. The ratio of surface to mass consequently decreases, but it is assumed that a constant cell density is maintained by an increase in cell diameter. It has furthermore been suggested that the change in diameter of cells which grow only in length under steady-state conditions is a physical response to the increase in turgor caused by the reduced differential rate of wall synthesis (18, 19, 28).

The simplest mechanism that might be anticipated to lead to a reduced differential rate of wall synthesis in response to an increase in growth rate would be a regulatory one affecting genes coding for an essential component of the cell envelope. Such a mechanism could not easily account for the analogous change in diameter that is shown here to occur when Thy$^-$ strains are grown on low-thymine media (Table 2), since in this case the composition of the growth medium is otherwise unchanged and the growth rate is not altered. It is this consideration that led initially to the suggestion that the reduced differential rate of envelope synthesis in low-thymine media and in which growth media might be a consequence of the change in chromosome configuration which occurs under both these conditions (28). It has been shown that the average number of copies of the chromosomes terminus per unit of mass will fall if the growth rate is increased or the replication velocity decreased. The average number of copies of the chromosome origin will be unaffected if the initiation mass is not changed in either case. The average number of copies of chromosome loci at intermediate positions will fall by intermediate amounts (see 19). Consequently, if the rate of envelope synthesis or the rate of length extension were proportional to the number of chromosome termini or to the number of copies of a gene located at any point distal to the chromosome origin, then a reduction in the differential rate of envelope synthesis would occur.

Detailed predictions relating cell shape and growth rate have been made for a number of models of this type (6, 18, 19, 27, 28; Grover et al., in press), and several of them are not incompatible with length and width data for a number of strains of *E. coli*. Quantitative comparison of the length and width data reported here for low- and high-thymine media with these models is given in the Appendix, and in several cases this consistency is maintained.

If the sole primary effect of thymine limitation is to reduce the rate of DNA chain elongation, then the associated change in cell width would constitute very strong evidence that the rate of envelope synthesis was coupled to chromosome replication, as the models evaluated in the Appendix assume. It must therefore be emphasized that we have not excluded the possibility that thymine limitation affects the rate of envelope synthesis directly. It has recently been reported (10, 11) that *E. coli* Thy$^-$ contains a large intracellular pool of a thymidine diphosphate sugar, the size of which fluctuates with the external thymine concentration. The possible role of similar compounds in the synthesis of envelope components (17) raises the possibility that thymine limitation might affect the rate of envelope synthesis directly. Until this possibility has been eliminated, we cannot conclude that a coupling between chromosome replication and the rate of envelope synthesis has been unambiguously established.

APPENDIX

Comparison of the changes in cell shape arising from a reduction in thymine concentration with the predictions of models

relating cell shape to the replication cycle. Several theoretical models, which assume that cell shape is influenced by the replication cycle, have been proposed to account for the changes in cell size and shape resulting from changes in growth rate. It is therefore of interest to make a quantitative comparison between the changes in cell length and width, which arise from a reduction in the concentration of thymine in the growth medium, with the predictions of the models for these conditions. The models assume that cell volume increases linearly, with discrete doublings in rate occurring once during each cell cycle, and that cell mass increases exponentially. The linear increase in volume has been attributed to either the existence of a finite number of growth sites and a constant rate of synthesis per site or to a constant rate of synthesis of a rate-limiting component of the envelope specified by an unregulated gene. Thus the doubling in rate occurs when the number of growth sites doubles or the number of copies of the gene doubles. The differences in average cell width, length, and hence surface area and volume under different cultural conditions are then accounted for by the differences in the average number of growth sites (or genes) per cell or by differences in the rate of extension per site (or output per gene), or by a combination of the two.

Model 1. Model 1 assumes that the number of sites for cell elongation corresponds to the number of chromosome termini per cell and that the linear rate of extension is proportional to growth rate (28). In a population of cells in steady-state exponential growth, the average number of termini per cell is $2^{D/\tau}$ (25) and average cell length, $\bar{L}$, is consequently given by the equation (27, 28):

$$\bar{L} = K_1 2^{D/\tau} \tag{1}$$

and cell width, $\bar{W}$, by (26):

$$\bar{W} = K_1 K_2 2^{C/2\tau} \tag{2}$$

where C, D, τ, K_1, and K_2 are, respectively, the chromosome replication time (4), the period between completion of a round of chromosome replication and cell division (4), the doubling time of the culture, the proportionality constant relating the rate of extension per site to the growth rate, and a constant incorporating the mass (or volume) of the cell at initiation of chromosome replication (5, 20).

Model 2. Model 2 proposes that the rate of envelope synthesis is proportional to the number of copies of a particular gene of which the product is a structural component of the envelope (18, 19). If the gene is located at a position, x, between the chromosome origin and terminus and the output of the gene is proportional to growth rate, then the average surface area per cell, SA, is given by the equation (19):

$$\overline{SA} = K_3 2^{[C(1-x)+D]/\tau} \tag{3}$$

where K_3 is the proportionality constant between gene output and growth rate.

Model 3. Model 3 is the "unit cell" hypothesis of Donachie and Begg (6), which postulates that the number of cell elongation sites is doubled at initiation of chromosome replication. Thus the average number of growth sites is equal to the number of chromosome origins per cell, $2^{(C+D)/\tau}$ (20). Elongation takes place from each site at a constant rate, K_4, which is independent of the growth rate, so that the average cell length, $\bar{L}$, is defined by the equation:

$$\bar{L} = K_4 2^{(C+D)/\tau} \cdot \tau \tag{4}$$

By assuming the cells to be regular cylinders ($V = \pi r^2 l$) with an average volume, $\bar{V}$, given by (5, 20):

$$\bar{V} = K_2 2^{(C+D)/\tau} \tag{5}$$

it follows that to a first approximation average cell width is given by:

$$\bar{W} = \sqrt{K_2/K_4\tau} \tag{6}$$

Model 4. Model 4, considered here for the first time, is a variant of model 3, in which it is assumed that the number of elongation sites equals the number of chromosome origins, but that the rate of extension per site is inversely proportional to the replication time C. In other words, the rate of cell elongation is coupled in some way to the rate of DNA chain elongation. In this case the equations for cell length, $\bar{L}$, and width, $\bar{W}$, become:

$$\bar{L} = K_4 2^{(C+D)/\tau} \cdot \tau C \tag{7}$$

$$\bar{W} = \sqrt{K_2 C/K_4\tau} \tag{8}$$

Analysis of cell length and width versus growth rate has shown a good agreement with the predictions of all of these models over the range of conditions where C and D are thought to remain approximately constant (4, 7, 19, 27, 28; Grover et al., in press). It should be noted in this context that the predictions of model 2 are only consistent with the experimental data if the gene is located just over halfway between the replication origin and terminus (19), and that models 3 and 4 give identical predictions about length and width changes if C and D are not affected by growth rate.

TABLE 3. *Comparison of predicted and observed changes in cell dimensions caused by a reduction in thymine concentration*

Determination	Change in:		
	Avg cell length	Avg cell width	Avg cell surface area
Predictions[a]			
Model			
1	0.86	1.33	
2 (i), $x = 0$			1.52
(ii), $x = 0.5$			1.14
(iii), $x = 1.0$			0.86
3	1.52	1.00	
4	0.84	1.34	
Observations[b]	0.90	1.32	1.19

[a] Calculated using the relevant equations from the Appendix based on C and D values for the 20- and 4-μg/ml cultures, respectively, of 41 and 22 and 74 and 13. The results are expressed as the ratio of the 4-μg/ml prediction to the 20-μg/ml prediction.

[b] Data from Table 2 expressed as the ratio of the 4-μg/ml measurement to the 20-μg/ml measurement.

Inspection of the equations reveals that all models predict changes in cell dimensions under conditions that alter C and D. We have therefore compared the change in cell length, width, and surface area produced by the step-down transition with those predicted by these equations, assuming the effect of the reduction in thymine concentration is to increase C from 41 to 74 min and to reduce D from 22 to 13 min. The data are clearly consistent with several of the models (Table 3). Only model 3 can be unambiguously excluded, since it predicts (equations 5 and 6) an increase in cell length and no change in cell width under the conditions of our experiment. These predictions are contrary to our observations. Model 2 is consistent with our data provided the "surface-limiting" gene is located approximately halfway along the replication sequence [Table 3, model 2(ii)].

Thus three models (1, 2(ii), and 4) are quantitatively consistent with the shape changes described here resulting from a decrease in thymine concentration, and those arise from an increase in growth as described by other authors.

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