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Changes in Cell Dimensions During Amino Acid Starvation of *Escherichia coli*

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Electron microscopic analysis was used to study cells of *Escherichia coli* B and K-12 during and after amino acid starvation. The results confirmed our previous conclusion that cell division and initiation of DNA replication occur at a smaller cell volume after amino acid starvation. Although during short starvation periods, the number of constricting cells decreased due to residual division, it appears that during prolonged starvation, cells of *E. coli* B and K-12 were capable of initiating new constrictions. During amino acid starvation, cell diameter decreased significantly. The decrease was reversed only after two generation times after the resumption of protein synthesis and was larger in magnitude than that previously observed before division (F. J. Trueba and C. L. Woldringh, J. Bacteriol. 142:869–878, 1980). This decrease in cell diameter correlates with synchronization of cell division which has been shown to occur after amino acid starvation.

In newly divided cells of *Escherichia coli*, the first division after amino acid starvation, as well as the first initiation of DNA replication, occurs earlier than in unstarved cells. Measurements of cell volume, performed with the Coulter Counter, as well as calculations of cell mass, indicate that these main events of the cell cycle occur long before the cells can increase their size to that at which these events occur in untreated cells (4, 11). In asynchronous, randomly dividing cultures of *E. coli* B and K-12, amino acid starvation results in a synchronous cell division soon after resumption of protein synthesis (12). The next round of DNA replication also starts in synchrony (unpublished data) and results in a few synchronized division cycles (12).

To understand how amino acid starvation brings about a shift forward in the subsequent cell cycles, we examined a few parameters which might play a role in the cell cycle and in induction of division. Important parameters in this respect are cell size and cell shape, which we examined by using electron microscopic measurements of bacteria obtained by the agar filtration technique (17). The results summarized in this communication confirmed our previous conclusion that after amino acid starvation, cells divide and initiate DNA replication when their size is smaller than that at which those events occur in untreated cells. In addition, the results indicated that during amino acid starvation of cells of *E. coli* B and K-12, there was a significant decrease in cell diameter. This decrease correlates with the synchronization of cell division which occurs at the end of the starvation, since it has not been observed in *E. coli* B/r, which could not be synchronized by amino acid starvation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used were *E. coli* B *ilvA thyA*; *E. coli* B/r H266 (our laboratory collection); *E. coli* B/r A *thr* (kindly supplied by A. Zaritsky) and its methionine-requiring mutant, B/r *thr met* (isolated in our laboratory after ethyl methane sulfonate mutagenesis and penicillin selection); and *E. coli* K-12 DG76 (F^- *thyA47 leu-6 str-163* [18], kindly supplied by B. Bachman) and K-12 428 *thi pro his* (from our laboratory collection).

Cells were grown with shaking at 37°C in minimal salt medium (6) supplemented with required amino acids (20 μg/ml) and thymine (20 μg/ml). The carbon source was added to a final concentration of 0.2%. At the start of the experiments, the cells had been growing exponentially for at least 10 generations.

Newborn cells of *E. coli* B were obtained by the membrane elution technique of Helmstetter (6). Newborn cells of *E. coli* K-12 were obtained by Percoll density gradient centrifugation (2).

Amino acid starvation. Amino acid starvation was initiated in one of the following ways: (i) addition of 5×10^{-4} M of the methionine analog α-methylmethionine (αMM) (Sigma Chemical Co., St. Louis, Mo.), which induces methionine starvation by inhibiting the first biosynthetic enzyme for methionine (14); (ii) addition of 5-methyltryptophan, which inhibits tryp-

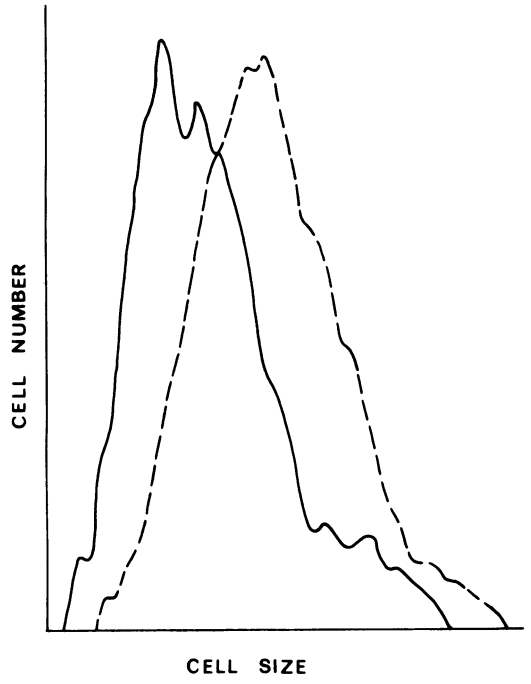

FIG. 1. Cell size distribution of *E. coli* B after amino acid starvation. Exponentially growing cells of *E. coli* B *ilvA* supplemented with glucose (0.2%) and isoleucine (20 μg/ml) were washed, suspended in prewarmed medium lacking the amino acid, and incubated at 37°C. Samples for size distribution were taken at the beginning (- - - -) and after 60 min (——) of starvation. The samples were fixed with formaldehyde (0.2%) and analyzed with a Coulter Counter (model ZB; 30-μm orifice).

tophan synthesis (10); (iii) addition of valine (100 μg/ml), which inhibits isoleucine biosynthesis in K-12 strains of *E. coli* (16); or (iv) removal of a required amino acid by filtrating through a Millipore filter (0.45 μm), washing with 10 times the volume, and suspending in prewarmed medium lacking the amino acid.

Cell counts. Bacterial concentration was determined with a Coulter Counter (model ZB; 30-μm orifice) after fixation with 0.2% formaldehyde.

Preparation of cells for electron microscopy. For electron microscopy, cells were fixed with 0.2% osmium tetroxide and air dried by a modification of the agar filtration technique described by Woldringh et al. (17). To promote spreading, tryptone (Difco Laboratories, Detroit, Mich.) was added to the fixed cells at a final concentration of 0.1%. Electron micrographs were taken with a JEM 100-B (Jeol, Japan) electron microscope at a 3,000× magnification.

Measurements of cell dimensions. Electron micrographs were projected at a final magnification of about 15,000× to 20,000×, and cell length and cell diameter were measured. At least 150 cells were measured for each sample, and dimensions were calculated according to a calibration grid which was photographed at the same magnification.

RESULTS

Effect of amino acid starvation on cell dimensions. The mean cell volume of exponentially growing cells of *E. coli* decreases during amino acid starvation. This decrease in cell size can be observed with a phase-contrast light microscope or by measuring cell volume with a Coulter Counter (Fig. 1). To quantitate the change in cell volume during and after amino acid starvation, we used the agar filtration technique (17) for determining cell dimensions by electron microscopy. The results presented in Fig. 2 demon-

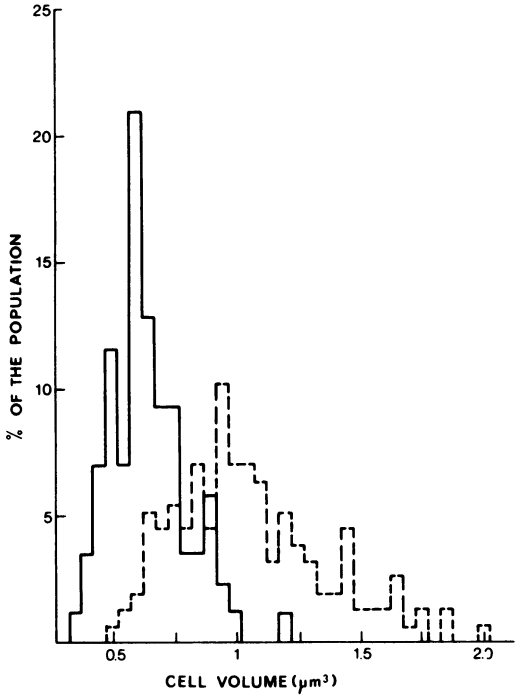

FIG. 2. Distribution of cell volume of *E. coli* B after amino acid starvation. Exponentially growing cells of *E. coli* B *ilvA thyA* supplemented with glycerol (0.2%) and isoleucine and thymine (20 μg/ml) were starved by the addition of αMM (5×10^{-4} M). Samples (1 ml each) were fixed with OsO_4 (0.2%) at the beginning (- - - -) and after 60 min (——) of starvation. After 2 min, tryptone (0.1%; Difco) was added, and the cells were pelleted by 2 min of centrifugation in an Eppendorf minifuge. The cells were washed and suspended in 0.5 ml of minimal salt medium containing azide (17) and osmium tetroxide (0.1%). Small drops of cells with tryptone (0.1%) were air dried by a modification of the agar filtration technique as previously described by Woldringh et al. (17). Cell length and cell diameter were measured on electron micrographs projected at a 20,000× magnification. At least 150 cells were measured for each point. Cell volume (V) for each cell was calculated according to the method of Trueba and Woldringh (15): $V = R^2 (L - 2/3R)$, where $2R$ = mean cell diameter and L = mean cell length.

TABLE 1. Effect of amino acid starvation on dimensions of *E. coli* B *ilvA thyA*[a]

Carbon source	Doubling time (min)	Type of starvation	Mean cell length (μm)[b]	Mean cell diam (μm)[b]	Mean cell vol (μm³)[c]
Glucose	50		2.72 (26.3)	0.98 (6.1)	1.804
		60 min +αMM	2.14 (21.7)	0.88 (5.3)	1.122
Glycerol	60		2.45 (25.0)	0.76 (5.3)	0.996
		60 min +αMM	2.00 (21.7)	0.66 (6.1)	0.609
Glycerol	60		2.27 (21.6)	0.77 (6.9)	0.937
		60 min −isoleucine	1.94 (18.8)	0.71 (5.4)	0.674

[a] Cells of *E. coli* B exponentially growing at different growth rates were starved for an amino acid. Samples (1 ml each) were removed at the beginning and the end of starvation and were treated as described in the legend to Fig. 2.
[b] Numbers in parentheses are the coefficients of variation [(standard deviation/mean) × 100].
[c] Calculated as described in the legend to Fig. 2.

strate a change in the frequency distribution of cell volume of *E. coli* B after 60 min of amino acid starvation. This pattern is indeed the same as that previously obtained with a Coulter Counter. The decrease in cell volume was independent of the previous growth rate of the exponentially growing cells or the amino acid for which they were starved (Table 1).

An important contribution to the decrease in cell volume comes from a significant decrease in the cell diameter of *E. coli* B and K-12 (Tables 1 and 2 and Fig. 3b) during amino acid starvation. The diameter decrease probably starts at the onset of starvation, since it can be detected even after the first 30 min of starvation. In contrast, no such drop in diameter was observed in several strains of *E. coli* B/r (Table 2). These strains did not show synchronous cell divisions after amino acid starvation (12).

Measurement of cell length also indicated a decrease in mean cell length (Table 1 and Fig. 3a). This decrease in cell length probably reflects residual cell division of 20 to 25% (as determined with the Coulter Counter) of those cells which have terminated DNA replication and are capable of division even in the absence of protein synthesis (1). This percentage comprises that fraction of the population which has entered the T period (T period is defined as the duration of the process of cell constriction) and started constriction before or at the onset of starvation (17). To further check this point, we studied the effect of amino acid starvation on the dimensions of newborn cells of *E. coli* obtained by the membrane elution technique (6). In these cells, division cannot occur in the absence of protein synthesis; indeed, under these conditions, no decrease in cell length was observed, whereas the decrease in diameter was comparable to that of asynchronously dividing cells

TABLE 2. Cell diameters of several strains of *E. coli* during amino acid starvation[a]

E. coli strain	Type of starvation	Mean cell diam (μm)[b]		
		Unstarved	Starved for 1 h	Starved for 2 h
DG76	+αMM	0.77 (6.1)	0.74 (4.8)	0.72 (5.6)
	−Leucine	0.76 (5.4)	0.73 (4.3)	0.72 (5.2)
	+Valine	0.77 (6.1)	0.73 (5.4)	0.72 (6.1)
428	−Proline	0.90 (4.6)	0.87 (4.1)	0.82 (4.8)
B/r H266	+5-Methyltryptophan	0.80 (4.8)	0.82 (5.0)	0.85 (5.4)
B/rA *thr*	−Threonine	0.76 (6.2)	0.75 (4.8)	0.77 (5.1)
B/rA *thr met*	−Threonine	0.75 (6.3)	0.96 (6.1)	0.79 (5.9)

[a] Exponentially growing cultures of several strains of *E. coli* were starved for an amino acid as described in the text. Samples of cells after different periods of starvation were processed for electron micrographs as described in the legend to Fig. 2.
[b] Numbers in parentheses are coefficients of variation (see footnote *b* of Table 1).

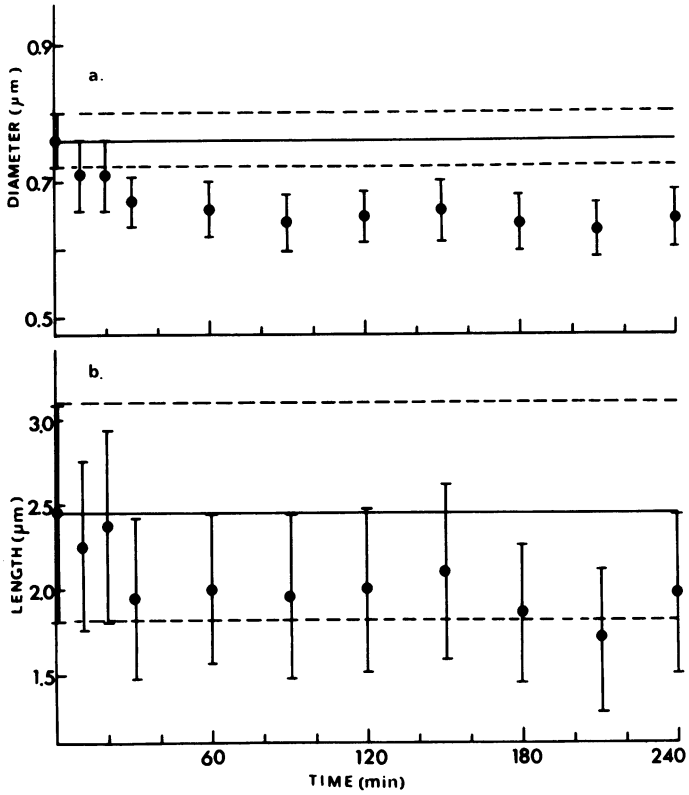

FIG. 3. Effect of amino acid starvation on mean cell diameter and cell length of *E. coli* B. Cultures of *E. coli* B *ilvA thyA* growing exponentially in glycerol salt medium (generation time = 50 min) were starved by the addition of αMM (5×10^{-4} M) when the cell concentration was about 5×10^7 per ml (zero time), and samples were removed at 30-min intervals. From the unstarved control, three consecutive samples were taken to ascertain balanced growth (17). Samples (1 ml each) were fixed with osmium tetroxide (0.2%) and treated as described in the legend to Fig. 2. The values for the unstarved control populations are shown as horizontal lines: the mean value is the solid line, and the dotted lines mark the standard deviation. Vertical lines indicate the mean values of the samples from the starved culture and the standard deviations.

(Table 3). Essentially the same results were observed during amino acid starvation of newborn cells of *E. coli* K-12 obtained by centrifugation in Percoll density gradients (2). These results suggest that the important change in cell dimensions which occurs during amino acid starvation is the decrease in cell diameter, which is independent of the age of the cell at the start of starvation.

The change in cell diameter was not immediately reversed. Rather, cell diameter stayed small for almost two generations after the resumption of protein synthesis (Fig. 4). As a result of the smaller cell diameter, cells coming out of starvation carried out two cell divisions as well as two initiations of DNA replication at a cell volume which was smaller than that in untreated cells. This finding was documented after starvation of exponentially growing cells (Table 4) and newborn cells (Table 5).

Cell constriction during starvation. It has been shown (12) that amino acid starvation of *E. coli* B and K-12 results in subsequent synchronization of cell division, with the first division occurring about 20 min after the addition of the amino acid. During the period of starvation, DNA replication is terminated, but the processes leading to division are blocked, possibly due to a requirement for protein synthesis (9). Because of residual division, the fraction of constricting cells decreases as a function of the length of starvation. However, after very long periods of starvation (longer than two generations), some escape could be observed, and the percentage of constricting cells increased (Table 6). In *E. coli* B/r, however, a more gradual decrease in the fraction of constricting cells was observed, and no new initiations of constriction occurred (Table 6).

The observation that in several strains of *E.*

TABLE 3. Dimensions of newborn cells of *E. coli* during amino acid starvation

Strain	Carbon source	Type of starvation	Cell length (μm)[a]	Cell diam (μm)[a]	Cell vol (μm^3)
E. coli B *ilvA thyA*[b]	Glycerol		1.61 (29)	0.73 (5.1)	0.572
		60 min +αMM	1.61 (22)	0.67 (7)	0.492
	Acetate		1.67 (26.9)	0.71 (9.6)	0.567
		90 min +αMM	1.81 (26.4)	0.66 (11)	0.544
E. coli K-12, DG-76[c]	Glucose		3.27 (27)	0.82 (3.5)	1.582
		60 min +valine	3.17 (28)	0.74 (3.5)	1.468

[a] Numbers in parentheses are coefficients of variation (see footnote *b* of Table 1).

[b] Newborn cells of *E. coli* B growing on various carbon sources were obtained by the membrane elution technique (6) as previously described (11). Samples (5 ml each) containing about 10^7 newborn cells per ml were divided in two equal parts, one of which was starved for methionine by the addition of αMM (5×10^{-4} M).

[c] Newborn cells of *E. coli* K-12 were obtained by Percoll density gradient centrifugation (2) and were immediately starved for isoleucine by the addition of valine (100 μg/ml).

coli (B and K-12) new constrictions could be initiated during prolonged starvation for amino acid (Table 6) shows that in these strains some processes can still proceed in nongrowing cells. For instance, it has been shown that in starved cells protein synthesis can continue at a low but significant rate (3). In addition, preliminary observations on *E. coli* B cells cultured in glucose medium and prepared by critical point drying for visualization of the nucleoids (17) showed that during prolonged starvation segregation of nucleoids continued (unpublished data).

DISCUSSION

Bacterial cells growing under various conditions have typical doubling times and typical cell dimensions (5, 13). These parameters reflect the coordination between the rates of mass increase, DNA replication, and envelope synthesis. We have previously shown (11, 12) that amino acid starvation uncouples this coordination. Thus, after amino acid starvation, cell division (11, 12) and initiation of DNA replication (4) occur long before the cells can grow and reach the expected size or expected ratio of mass to DNA presumably required for division and initiation of DNA replication.

In this study, we used electron microscopic measurements of cells obtained by the agar filtration technique (17) to quantitate the change in cell size and cell shape during and after amino acid starvation. The results confirmed our previous conclusions and showed that during amino acid starvation, cell volume decreases; it remains small after starvation, so that both cell division and initiation of DNA replication occur at a cell volume smaller than usual.

Experiments performed with exponentially growing cells of *E. coli* B and K-12 showed that the decrease of cell volume during starvation

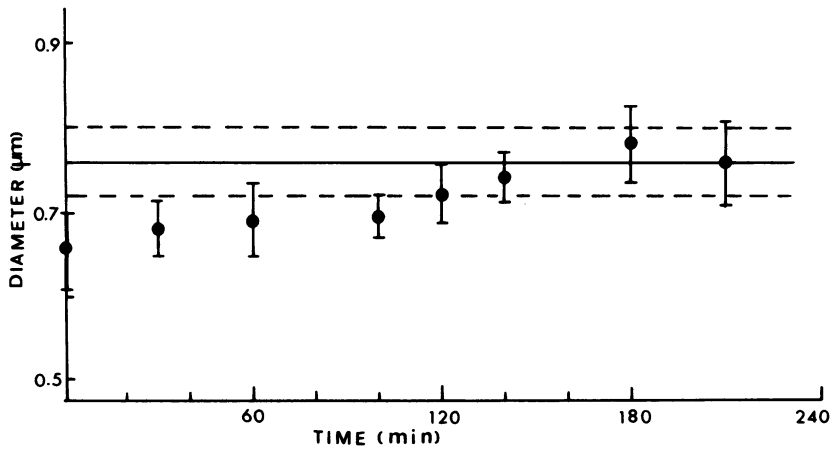

FIG. 4. Increase of cell diameter after amino acid starvation of *E. coli* B. Cells were treated as described in the legend to Fig. 3. After 60 min of starvation, methionine (100 μg/ml) was added (zero time), and samples were taken and processed as described in the legend to Fig. 3. For symbols, see the legend to Fig. 3.

TABLE 4. Dimensions of constricting cells at the synchronous divisions after amino acid starvation of exponentially growing cultures of *E. coli* B

Sample	Timing of division (min)[a]	Mean cell length (μm)[b]	Mean cell diam (μm)[b,c]	Mean cell vol (μm³)	Vol relative to a newborn cell[d]
Unstarved		3.22 (14)	0.79 (5.3)	1.45	1.88
During division 1	20	2.90 (13.8)	0.71 (5.8)	1.05	1.36
During division 2	80	3.16 (14.1)	0.68 (5.6)	1.07	1.39
During division 3	135	3.07 (16.5)	0.75 (2.8)	1.24	1.62

[a] The timing of synchronous division is the time of 50% increase of cell concentration after the end of starvation.
[b] Numbers in parentheses are coefficients of variation (see footnote *b* of Table 1).
[c] The mean cell diameter at the end of starvation was 0.66 μm (coefficient of variation = 6.1).
[d] The mean cell volume of a newborn cell was calculated by the method described in the legend to Fig. 2 and found to be 0.77 μm³.

was accompanied by a significant decrease of cell diameter as well as a decrease in cell length. The decrease in cell length probably reflects residual division of those cells which divide in the absence of protein synthesis, as indicated by the decrease in the percentage of constricted cells (Table 6).

The decrease in cell diameter was, however, more marked and was the only change that occurred, even during starvation of newborn cells, in *E. coli* B and *E. coli* K-12. Moreover, this decrease was independent of cell age, growth rate before starvation, and the amino acid for which the cells were starved.

The molecular and biochemical basis for the decrease in cell diameter and the initiation of new constrictions during amino acid starvation are still not understood. Preliminary studies suggest that the structure of peptidoglycan is involved, since a comparable decrease in dimensions can be demonstrated in murein sacculi (unpublished data). A decrease in cell diameter is unusual, since other known treatments which delay cell division, such as thymine limitation (19), bring about an increase in cell diameter. A decrease in cell diameter is also not easy to understand in view of the rigidity of murein. The decrease in cell diameter might reflect a change in the degree of cross linkage in the murein (R. E. Harkness and E. E. Ishiguro, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, K199, p. 159) or another structural change and could be a result of the stringent control of murein synthesis (7, 8) which is induced by amino acid starvation.

Recently, Trueba and Woldringh (15) have found that cell diameter decreases with progress through the cell cycle until constriction has been initiated and cell diameter increases again. The decrease in cell diameter that is observed during

TABLE 5. Dimensions at division and initiation of replication after the end of starvation of newly divided cells of *E. coli* B

Carbon source	Type of starvation	At division				At initiation of replication	
		Timing of division (min)[a]	Mean cell length (μm)[b]	Mean cell diam (μm)[b]	Cell vol (μm³)[c]	Timing of initiation (min)[a]	Mean cell vol of whole population (μm³)
Glycerol		50	2.57 (19.6)	0.75 (3.3)	1.025	40	0.720
	60 min +αMM	20	2.43 (5.4)	0.69 (3.6)	0.819	10	0.488
Acetate		104	2.74 (7.6)	0.72 (9.6)	1.018	91	0.761
	90 min +αMM	52	2.54 (12.7)	0.65 (11.1)	0.771	39	0.633

[a] The timing of division or initiation is the time of 50% increase of cell concentration or rate of replication.
[b] Numbers in parentheses are coefficients of variation (see footnote *b* of Table 1).
[c] Cell volume was calculated according to the method of Treuba and Woldringh (15), as described in the legend to Fig. 2. Newborn cells of *E. coli* B growing on various carbon sources were obtained by the membrane elution technique (6) and were treated as described in footnote *b* of Table 3. Samples for determination of cell concentration and rate of replication were taken every 10 and 13 min for glycerol- and acetate-grown cells, respectively, as described previously (4).

TABLE 6. Effect of amino acid starvation on cell constriction of *E. coli* B and B/r[a]

E. coli strain	Carbon source	Type of starvation	% Constricting cells				
			Unstarved culture	During starvation of (min): 60	120	180	At division 1 after 60 min of starvation
B *ilvA thyA*	Glucose	+αMM	19.9	4.4	8.3	16.6	34.5
B *ilvA thyA*	Glycerol	+αMM	16	6	5	11	23.6
B/r H266	Glucose	+5-Methyltryptophan	19.4	10.4	4.4	2.4	—[b]
B/r *thr*	Glucose	−Threonine	17.1	10	9.3	1.1	—

[a] Exponentially growing cells of *E. coli* B and B/r were starved as described in the text. At least 300 cells were scored for constriction in electron micrographs as described by Treuba and Woldringh (15).

[b] —, Cell constriction could not be determined in *E. coli* B/r after starvation, because the division is asynchronous.

amino acid starvation might result from mechanisms similar to those responsible for the decrease before constriction, yet it is different in at least two aspects. The decrease in diameter during amino acid starvation is larger in magnitude (up to twofold) than the decrease before division, and it takes almost two generations before cell diameter increases and returns to that of unstarved control cells.

Nevertheless, a decrease in cell diameter could be a prerequisite for the triggering of cell division or, otherwise, a condition which facilitates division. Thus, cells of *E. coli* K-12 and B which are capable of continuing preparations for division during amino acid starvation, as indicated by the increase in the percentage of cells which have initiated constriction, divide synchronously upon resumption of protein synthesis (12). This behavior was not shown by *E. coli* B/r cells (Tables 2 and 6), which could not be synchronized by amino acid starvation (12). In these cells, both the decrease in cell diameter and the initiation of new contrictions progressed very slowly during amino acid starvation.

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