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Growth Rate-Dependent Control of Chromosome Replication Initiation in *Escherichia coli*

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The initiation mass, defined as cell mass per origin of deoxyribonucleic acid replication (optical density units at 460 nm of culture/origins per milliliter of culture), reflects the intracellular concentration or activity of a hypothetical factor that controls initiation of chromosome replication in bacteria. In *Escherichia coli* B/r, the initiation mass was found to increase about twofold with increasing growth rate between 0.6 and 1.6 doublings per h; at higher growth rates it remained essentially constant (measured up to 2.4 doublings per h). A low-thymine-requiring (*thyA deoB*) derivative of *E. coli* B/r, strain TJK16, was found to have a 60 to 80% greater initiation mass than B/r which was independent of the replication velocity and not related to the *thyA* and *deoB* mutations. It is suggested that TJK16 had acquired, during its isolation, a mutation in a gene affecting the initiation of deoxyribonucleic acid replication. The initiation age was not altered by this mutation, but other parameters, including deoxyribonucleic acid concentration and cell size, were changed in comparison with the B/r parent, as expected from theoretical considerations.

Donachie (13) defined the initiation mass as cell mass at initiation of a round of replication divided by the copy number of replication origins present at that time. By combining Helmstetter and Cooper's (21) estimates of the C and D periods (C is the time to replicate a chromosome, equal to the time between initiation and termination of a round of replication; D is the time between termination and subsequent cell division) in *Escherichia coli* B/r with estimates by Schaechter et al. (32) of the average cell mass of *Salmonella*, Donachie was able to calculate the initiation mass and found it to be independent of the rate of growth. From this growth rate invariance, he concluded that the initiation mass may be an important parameter for the control of initiation in bacteria. We show here for *E. coli* B/r that the initiation mass is not growth rate invariant, at least not in slow-growing bacteria. However, that fact does not diminish the significance of the initiation mass for the control of initiation: the variable initiation mass is an expression of a growth rate-dependent control of the synthesis of initiation control factor (see Discussion).

To determine the initiation mass, we used a method (3) that does not require knowledge of the initiation age and which is simpler and potentially more accurate than the method used by Donachie. In fact, the initiation age can be shown to be irrelevant for the control of initiation (see Discussion).

In the experiments reported below, we also used a low-thymine-requiring derivative of *E. coli* B/r, TJK16, a strain that we had previously used to measure the C period at different thymine concentrations and growth rates (2, 4, 9). Unexpectedly, this strain was found to have a 60 to 80% greater initiation mass than *E. coli* B/r. In the accompanying paper (8), we show that this strain had acquired, in addition to the *thyA* and *deoB* mutations that had been selected to obtain a low-thymine requirer, a mutation which affected the initiation mass. This mutation is in the *dnaA* region of the chromosome (1, 8).

The initiation mass is one of the factors that determine the cell size and DNA concentration. The relations between these parameters are discussed below.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used were *E. coli* B/r A (21; ATCC 12407) and a low-thymine-requiring *thyA deoB* derivative, TJK16, isolated by J. Kwoh (Ph.D. thesis, University of Texas at Dallas, 1980). They were grown at 37°C in minimal medium C (17) supplemented with 0.2% of either succinate, glycerol, or glucose or with glucose plus 20 L-amino acids, each to a concentration of 50 μg/ml. Cultures of TJK16 contained, in addition, 1 or 20 μg of thymine per ml as indicated in the figure legends.

Determination of cell mass, DNA, protein, and cell number. Cell mass was determined as optical

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density at 460 nm (OD_{460}) of the culture (1-cm light path). Cell number was determined by using a Coulter Counter (model B; 20-μm orifice) equipped with a Coulter Channelyzer after dilution of samples in 0.9% saline containing 4 ml of 37% formaldehyde per liter. For volume distributions, the "edit" function was turned on. The methods for determining DNA, RNA, and protein have been described elsewhere in detail (5). Duplicate samples were taken at an OD_{460} of approximately 0.3 and 0.6. Briefly, for DNA, 10-ml samples were precipitated with 2 ml of cold 3 M trichloroacetic acid, filtered through glass fiber filters (Reeve Angel; 984H), washed with tap water, dried, and placed in glass vials. After hydrolysis in 1.0 ml of 1.6 M $HClO_4$ at 23°C (rather than at 70°C as previously) for 30 min, the DNA was estimated colorimetrically by a modification of the Burton method (6). Under these conditions, an absorbancy at 600 nm (A_{600}) of 1.0 corresponds to 2.34×10^{16} nucleotides or 2.84×10^{9} genome equivalents per ml of culture (calibration with purified *E. coli* DNA, assuming 8.2×10^{6} nucleotides per genome [1]). Protein was determined by a modification of the method of Lowry et al. (23) as described by Brunschede et al. (5), using 1.25-ml samples of bacterial culture, The protein assays were incubated for 20 h at 23°C. Under these conditions, an A_{750} of 1 corresponds to 9.1×10^{17} amino acids per ml of culture (calibration with bovine serum albumin).

RESULTS

Initiation mass of *E. coli* B/r and TJK16. The initiation mass (M_o) was determined from mass (OD_{460}) and DNA measurements of bacterial cultures growing at different rates (Fig. 1b; M/G is mass per genome) and from the increase in DNA (ΔG) after inhibition of initiation (e.g., with chloramphenicol [Fig. 1a]; values plotted are from Tables 1 and 2 of reference 9). The value of ΔG is equivalent to the number of replication origins per genome (3); hence, it is seen that mass per origin is equal to mass per genome divided by origins per genome [$M_o = (M/G)/\Delta G$].

The ΔG values were the same for B/r and TJK16, growing with 20 μg of thymine per ml. In TJK16, ΔG increased with decreasing thymine concentration in the medium (Fig. 1a). At a given growth rate, different ΔG values reflect differences in the replication velocity; the relationships between ΔG and replication velocity are well understood (2, 4, 15, 27, 30, 33).

Mass per genome was greater for TJK16 (at 20 μg of thymine per ml) than for B/r, and it was even greater when TJK16 was grown at the low thymine concentration of 1 μg/ml (Fig. 1b). Combining the mass values of Fig. 1b with the ΔG values of Fig. 1a gave the initiation mass for B/r and TJK16 as a function of growth rate: in either strain it increased with increasing growth rate about twofold between 0.6 and 1.6 to 2 doublings per h, but in TJK16 the initiation mass was always 60 to 80% greater than in B/r

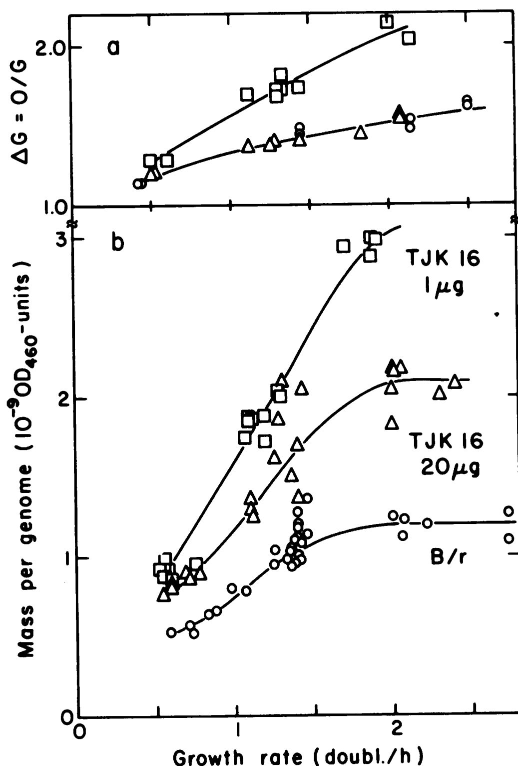

FIG. 1. *(a) Increase in DNA (ΔG) after inhibition of initiation of replication with chloramphenicol; data plotted from Tables 1 and 2 of reference 9. The increase is given as factor (>1); i.e., ΔG = 1.4 means a 1.4-fold (= 40%) increase. (b) Cell mass per genome. Each point represents the average of four DNA measurements made on one culture at OD_{460} ~ 0.3 (two samples) and 0.6 (two samples). Symbols: ○, E. coli B/r; Δ, TJK16 (20 μg of thymine per ml); □, TJK16 (1 μg of thymine per ml).*

(Fig. 2), suggesting that TJK16 has a mutation that affects the control of initiation (see below). In TJK16, the initiation mass was independent (or essentially independent) of thymine concentration; i.e., initiation was independent of thymine metabolism and of the replication velocity, as has been concluded previously (4, 30).

Protein per origin. An alternate measure for the control of initiation is the amount of protein per origin (P_o); it may be obtained (analogously to M_o) from measurements of protein per genome (Fig. 3b) and ΔG (Fig. 1a), or from M_o by multiplication with protein per mass (Fig. 3c). Due to the scatter in the data points, representing both variations in the reproducibility of protein and DNA assays (±6% for protein and ±3% for DNA) and true variations from culture to culture, exact P_o curves could not be obtained for TJK16. However, since the protein-per-mass data for the two strains were the same, P_o shows

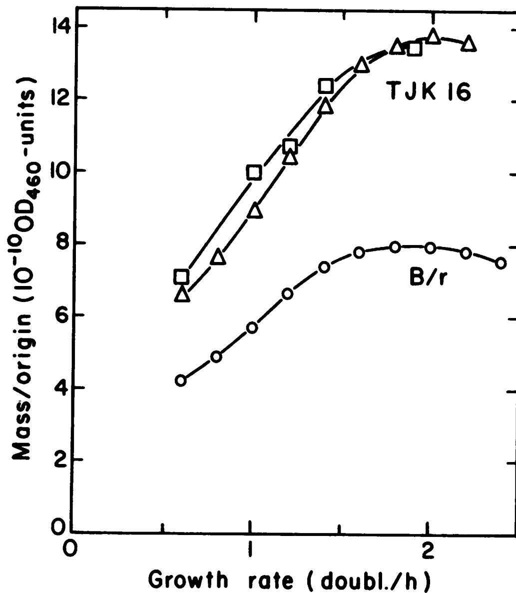

FIG. 2. *Cell mass per origin of replication for E. coli B/r and TJK16, obtained from the data in Fig. 1 (mass per origin = mass per genome + ΔG). Symbols are as in Fig. 1. The points are not directly observed, but taken from the curves in Fig. 1.*

the same strain differences as M_o, but the growth rate dependence of P_o is somewhat less than that of M_o (Fig. 3a).

Cell mass and volume. At a given growth rate, the average cell mass ($\bar{M}$) is determined by three parameters, one of which is the initiation mass; the other two are the C and D periods (see Discussion). Therefore, the greater initiation mass of TJK16 can be expected to result in greater average cell mass and volume. This was in fact observed (Fig. 4, cell volume distribution; Table 1, average cell mass, $\bar{M}$, and average cell volume, $\bar{V}$).

DISCUSSION

Measurement of the initiation mass. Donachie (13) defined the initiation mass, M_i, as mass per origin in single cells at the time of initiation. Our definition of initiation mass is "mass per origin in exponential cultures," denoted by the symbol M_o; the average M_i in a population, $\bar{M}_i$, and M_o differ by a constant factor (equal to ln2; see reference 3). M_i cannot be directly measured. Indirectly, Donachie (13) has calculated relative values of $\bar{M}_i$ in exponentially growing populations of bacteria by combining published measurements of the average cell mass in *Salmonella* cultures (32) with estimates of the average initiation age in *E. coli* B/r (19). Similar calculations have been reported elsewhere (20). These calculations suggested that the average initiation mass is nearly independent of growth rate.

By combining radioautographic data from bacteria pulse-labeled with [^{3}H]thymidine with electron microscopic analyses of the variation in cell lengths, Koppes et al. (22) have estimated for three substrains of *E. coli* B/r frequency distributions of cell lengths at birth, at initiation and termination of DNA replication, and at initiation of cell constriction and separation. From such data, those authors calculated the average volume of the cells at initiation, $\bar{V}_i$, a parameter related to $\bar{M}_i$. Since autoradiographic data were used for determining the beginning of DNA replication, the measurements could only be done with slowly growing bacteria in which there is a gap between consecutive rounds of replication (10). Therefore, these data do not contribute to the question of the growth rate dependence of $\bar{V}_i$. From measurements of DNA synthesis and mean cell volumes in synchronous cultures, Helmstetter (18) found that slowly growing cells have a 30% smaller $\bar{V}_i$ than rapidly growing cells.

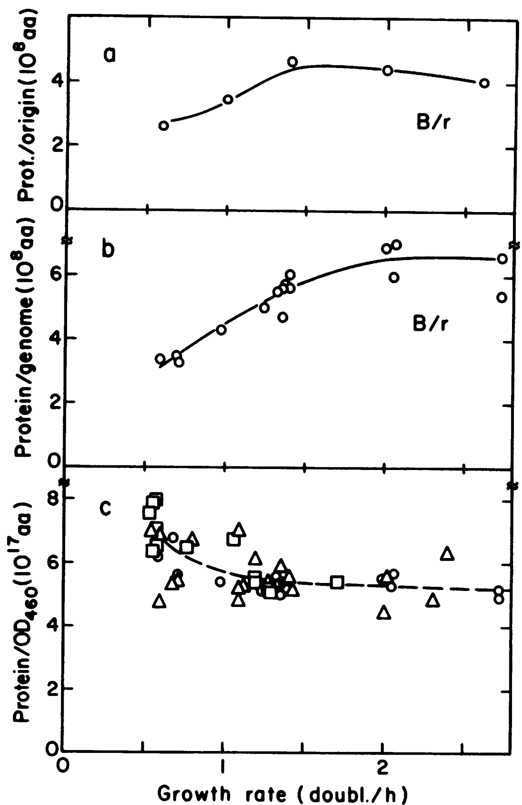

FIG. 3. *Protein per origin (a), per genome (b), and per OD_{460} (c) for E. coli B/r (a–c; ○) and for TJK16 (only panel c; □, △, 1 and 20 μg of thymine per ml).*

Since the volume per mass (V/M) decreased with the growth rate (Table 1), $\bar{M}_i$ must vary even more with the growth rate, in qualitative agreement with our data in Fig. 2.

Based on a thoretical study (3), we measured M_o in *E. coli* B/r and in a thymine-requiring derivative, TJK16, which we previously used to determine the replication velocity (9). The main results were (i) the initiation mass increased with growth rate, at least at growth rates below 1.5 doublings/h; (ii) the thymine-requiring derivative, TJK16, had an increased initiation mass in comparison with *E. coli* B/r at all growth rates; (iii) initiation age and rate were the same for *E. coli* B/r and TJK16; (iv) cell size and DNA concentration were different for the two strains. The significance of these findings is discussed below.

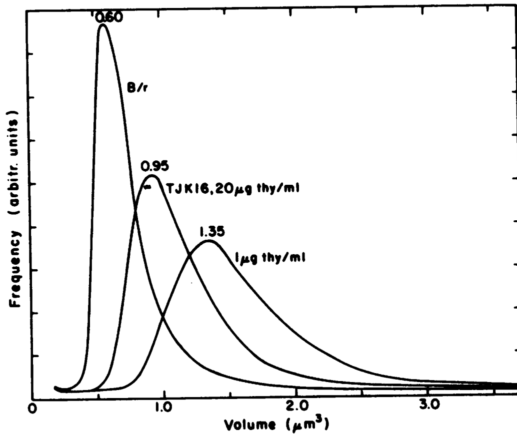

FIG. 4. *Cell volume distributions of E. coli B/r and TJK16 grown in glucose minimal medium to OD_{460} = 0.6. Peak volumes indicated were used to estimate the average cell volume (Table 1). Each distribution represents 10^4 cells.*

For the determination of M_o, we used our previously measured values of ΔG (9), which imply that the C period decreases continuously from 60 to 40 min with increasing growth rate in the range of growth rates used here (9). With different techniques, it has been determined that under these conditions C remains constant in *E. coli* K-12 (7). If in fact C were constant and equal to 40 min also in slowly growing B/r, this would tend to slightly increase the values of P_o and M_o at the lower growth rates. For a growth rate of 0.67 doublings/h, P_o would become equal to 2.9×10^8 rather than 2.7×10^8 amino acid residues per origin. Neither parameter would become constant and independent of growth rate.

Significance of the growth rate dependence of the initiation mass. From his finding

TABLE 1. *Cell size and related parameters of E. coli B/r and TJK16*

Strain	Medium	Thymine (μg/ml)	τ (min)	C[a] (min)	O/G[b]	G/M[c]	$\bar{M}$[d]	$\bar{G}$[e]	M_o[f]	$\bar{V}$[g] (μm³)	V/M[h] (μl/ OD_{460})	C + D[i] (min)	D[j] (min)
B/r	Glucose	0	46	46	1.40	1.00	2.4	2.4	0.72	0.83	0.35	79	31
TJK	Glucose	20	46	48	1.40	0.63	3.8	2.4	1.13	1.32	0.34	81	33
TJK	Glucose	1	46	81	1.73	0.45	5.9	2.7	1.28	1.90	0.33	101	20
B/r	Glucose + AA[k]	0	28	38	1.54	0.80	3.8	3.0	0.81	1.12	0.29	62	24
TJK	Glucose + AA	20	28	39	1.56	0.45	6.6	3.0	1.42	1.70	0.26	62	23
TJK	Glucose + AA	1	32	79	2.09	0.33	9.2	3.0	1.45	2.20	0.24	85	6

[a] Calculated from ΔG and τ, using $\Delta G = n \ln 2/(1 - 2^{-n})$; $n = C/\tau$ (ΔG, from Fig. 1a).

[b] Origins/genome = ΔG, from Fig. 1a.

[c] DNA/mass in 10^9 genome equivalents/OD_{460} unit, from Fig. 2b (reciprocal of M/G).

[d] Average cell mass = OD_{460} units/10^9 cells.

[e] Genome equivalents/cell = $G/M \times \bar{M}$.

[f] Mass per origin, obtained from G/M and O/G, $M_o = 1/[(G/M)\cdot(O/G)]$ in 10^{-9} OD_{460} units/origin.

[g] Average cell volume, estimated from most frequent cell volume (peak in the volume distributions of Fig. 4 and from a similar experiment using glucose-amino acid-grown cells) by multiplication with 2 ln2 = 1.39.

[h] Volume/mass in μl/OD_{460}-unit (i.e. the space that one OD_{460}-unit of cells would assume) obtained as the ratio $\bar{V}/\bar{M}$.

[i] Sum of C and D periods obtained from $\bar{M}$, M_o, and τ, using the equation $\bar{M}/M_o = 2^{(C+D)/\tau}$ = average number of origins per cell.

[j] D period obtained as the difference (C + D) − C. The D period of TJK16 is here seen to be not significantly different from the D period in B/r. However, in TJK16 the D period is somewhat variable and often is somewhat increased in comparison with B/r.

[k] Glucose plus amino acids (20 amino acids).

that the average initiation mass is approximately growth rate invariant, Donachie (13) speculated that there is also a constancy of initiation masses in the individual cells within a population of exponentially growing bacteria. This constancy of initiation masses within a cell population is not implied in the growth rate invariance of the population average; rather, the invariability from cell to cell and the variation of the average initiation mass with growth rate are separate phenomena with different meanings: the invariability from cell to cell relates to models of initiation control, whereas the variation with growth rate relates to the regulation of synthesis of the controlling element (see below).

The idea of a constant initiation mass within a cell population is experimentally supported by measurements of the variability of cell volumes at initiation, which is considerably less than the variability of the cell volumes at division (22). Indirectly this idea is also supported by the findings of relatively small variations of the time periods between consecutive replications of any given segment of the bacterial chromosome in exponential cultures of *E. coli* (25, 26; H. Bremer and L. Chuang, J. Theor. Biol., in press). Our measurement of the growth rate dependency of the average initiation mass does not contribute to this question of the constancy or variability of initiation masses within a cell population.

The concept of initiation mass constancy has been incorporated into two models of control of initiation of bacterial DNA replication. (i) The positive control model (20, 28) assumes that a factor, limiting the number (not the rate) of initiations, accumulates during cell growth. (ii) The negative control model (29) assumes that all requirements for initiation are present in excess (i.e., not in rate-limiting quantities); however, initiation is inhibited by a repressor that is made in bursts immediately following an initiation event. No decision in favor of one or the other hypothesis has been possible. (Perturbation analysis, the major tool that has been used, leads to formally the same predictions for both models [14].) For positive control, the initiation mass represents the reciprocal of the concentration or activity of the initiation protein; for negative control, the initiation mass represents a measure for concentration (or activity, or both) of the initiation repressor. Other models assume that initiation of DNA replication is triggered by events involving cell surface growth (12, 18). In those models, M_o reflects a surface/origin relationship related to the fact that the bacterial chromosome is attached to the bacterial membrane at or near the site of the replication origin.

Whatever factor is limiting initiation of chromosome replication, i.e., whether it be a positive factor or a repressor, synthesis of this factor might be under some form of control to prevent "runaway replication," which would result in small cells with an excessive amount of DNA in comparison with other cell components (such as ribosomes) or "under-replication," that is, large cells with a DNA content so low that it would limit the rate of transcription. But the control does not have to be very strict since the cell can apparently tolerate at least twofold variations in cell size and DNA concentration without appreciable effects on the rate of growth, as shown by the comparison of B/r and TJK16 (Table 1). If the synthesis of initiation factor were controlled, then some growth rate dependency of this control would not be surprising.

Independent of whether the control of initiation is positive or negative, synthesis of the control factor, i.e., of initiation protein (positive) or initiation repressor (negative), might be controlled negatively, by a repressor. Particularly, the control factor might control its own synthesis by acting as an autorepressor. Such an autoregulation has been suggested for the *dnaA* protein (16). If *dnaA*(Ts) strains are grown at intermediate temperatures when the *dnaA* protein is partially inactivated, initiation of rounds of replication is reduced (16), suggesting that, under these semipermissive conditions, the amount of active *dnaA* protein limits the number of initiations (not the initiation rate) in a manner as postulated by the positive initiation model.

It is also conceivable that the factor is synthesized constitutively without a special control involving an operator site near the promoter. In that case, the growth rate dependency of the initiation mass could be the result of indirect or metabolic regulation (31). For example, if initiation depended on a positive factor, the observed increase of the initiation mass with growth rate would indicate a decreasing synthesis of initiation factor per total protein with increasing growth rate. Such a behavior is also found for constitutive *lac* gene expression; i.e., constitutive β-galactosidase enzyme per total protein decreases with growth rate about twofold between 0.6 and 1.5 doublings/h and remains approximately constant at higher growth rates (11). This is similar to the growth rate dependency of the initiation mass observed in Fig. 2 (note that the initiation mass reflects the reciprocal of the specific activity of a hypothetical positive initiation factor).

TJK16 has an altered control of replication initiation. The increased initiation mass of TJK16 compared with B/r persists in a *thyA*$^+$ *deoB*$^+$ double revertant of TJK16 and is not observed in other, independently isolated *thyA deoB* derivatives of B/r (8). This indicates that

TJK16 contains a mutation in a gene that affects the control of initiation of replication. This mutation is characterized genetically in the accompanying paper (8), which shows that this mutation maps in the *dnaA* region of the chromosome and probably is located in the *dnaA* gene. The identification of a new spontaneous initiation control mutant was facilitated by the availability of a technique to measure the initiation mass without age fractionation.

Initiation age and rate are the same for *E. coli* B/r and TJK16. The timing of initiation during the cell cycle (initiation age) is a function of C, D, and τ (10, 19). Since these parameters have essentially the same values for *E. coli* B/r and TJK16 grown at high thymine concentrations (Table 1), at a given growth rate and at high thymine concentrations, B/r and TJK16 must have the same initiation age. The rate of initiation may be defined as rate per origin, per genome, or per cell. With either definition, the rate is a function of C, D, or τ, or all three (3); i.e., it also is the same for B/r and TJK16. Thus, in spite of the quantitatively different control of initiation of DNA replication in these two strains, neither the timing nor the rate of initiation is affected by this control. This apparent paradox is explained as follows.

Although C, D, and τ determine the initiation age and rate, the control mechanisms of these parameters have no direct relation to the control of initiation. (The mass doubling time τ [reciprocal of the growth rate] reflects the control of ribosome synthesis [24], C [reciprocal of replication velocity] reflects the control of deoxyribonucleotide pools [30], and D reflects the control of cell division.) Therefore, the altered control of initiation in TJK16 shows itself in an altered initiation mass but not in an altered initiation age. This finding supports the notion that the time of initiation with respect to the preceding division is not controlled; rather, initiation is controlled with respect to some parameter that relates to mass increase.

Cell size and DNA concentration of B/r and TJK16. The different cell volumes (Fig. 4) and DNA concentrations (Fig. 1b; mass per genome is the reciprocal of the DNA concentration) for *E. coli* B/r and TJK16 growing at different rates or at different thymine concentrations, respectively, can be explained from theoretical equations which relate $\bar{M}$ or G/M, respectively, to M_o, C, D, and τ (12, 30). The average cell mass, $\bar{M}$, is given by (3, 12):

$$\bar{M} = M_o \cdot 2^{(C + D)/\tau}$$

Accordingly, $\bar{M}$ must increase in proportion to M_o, which is the reason for the larger cell volume of TJK16 (at 20 μg of thymine per ml) compared with B/r (Fig. 4). The cell mass must also increase (exponentially) with increasing duration of the C period, which is consistent with the further increase of the cell volume found when TJK16 is grown at the lower thymine concentration of 1 μg/ml (Fig. 4). Furthermore, $\bar{M}$ must increase with $1/\tau$ (Table 1).

The DNA concentration, expressed as genome equivalents of DNA per unit of mass, G/M, is given by (30):

$$G/M = \frac{1 - 2^{-C/\tau}}{M_o(C/\tau)\ln 2}$$

Accordingly, TJK16 growing at high thymine concentrations has a lower DNA concentration than B/r because of increased M_o; the DNA concentration decreases even more if TJK16 is grown at low thymine concentrations because of increased C (Fig. 1b). B/r and TJK16 grow at the same rate (Table 1), which shows that the DNA concentration is not a growth-limiting factor.

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