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Increase in Cell Mass during the Division Cycle of *Escherichia coli* B/rA

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Increase in the mean cell mass of undivided cells was determined during the division cycle of *Escherichia coli* B/rA. Cell buoyant densities during the division cycle were determined after cells from an exponentially growing culture were separated by size. The buoyant densities of these cells were essentially independent of cell age, with a mean value of 1.094 g ml^{-1}. Mean cell volume and buoyant density were also determined during synchronous growth in two different media, which provided doubling times of 40 and 25 min. Cell volume and mass increased linearly at both growth rates, as buoyant density did not vary significantly. The results are consistent with only one of the three major models of cell growth, linear growth, which specifies that the rate of increase in cell mass is constant throughout the division cycle.

During the past two decades there have been a greater number and variety of studies of cell growth with *Escherichia coli* than with any other procaryotic or eucaryotic cell type (3, 4, 22, 24, 28). These studies have included measurement of cell length in individually growing cells by optical microscopy (4, 7, 25), measurement of cell length or volume in synchronized cultures (9, 13, 20, 26, 29), and analysis of distributions of cell length or volume in populations isolated from exponential-phase cultures (2, 6, 10, 11, 16, 19). Different growth kinetics have been observed for *E. coli* with each of these approaches.

None of these studies described cell growth in terms of increase in the fundamental parameter cell mass. On the average, cell mass must double from birth to division during steady-state growth in a culture. But this requirement need not apply precisely to cell length or volume, both of which are critically dependent on cell shape, which is altered at division. Cell volume is only proportional to cell mass during the growth cycle when cell buoyant density is constant. On the other hand, cell mass may be thought of as an indicator of the number of molecules needed to sustain a self-replicating system. Because mass is the fundamental parameter of cell growth, I have examined the increase in cell mass during the division cycle of *E. coli* in the well-studied strain B/rA.

MATERIALS AND METHODS

Growth conditions. *E. coli* B/rA (ATCC 12407) was cultured in a shaker water bath at 37°C in Erlenmeyer flasks (250 ml) containing 30-ml volumes of MSGT medium (M9 salts, 20 g of sucrose and 4 g of glucose per liter, and 0.01% Hoagland trace element salt solution [1] without selenium), or in MSGT-CAA medium (MSGT medium with Casamino Acids [1 g/liter] added). The addition of the sucrose was required to prevent perturbation of cell growth by osmotic shock during later selection of cells in sucrose gradients (13). Cultures were prepared weekly by inoculating these media with a single colony from nutrient agar and allowing growth to the stationary phase before refrigeration. To obtain cultures in the exponential phase of growth, I diluted the weekly refrigerated cultures 10^5- to 10^6-fold during inoculation into the same medium the evening or night before use. Growth was measured as the increase in turbidity with a Klett-Summerson colorimeter provided with a Kodak 66 filter.

Preparation of synchronous cultures. Synchronous cultures were prepared by selecting cells from exponentially growing cultures by velocity sedimentation in sucrose gradients. Cells were concentrated by filtration and resuspension and banded by centrifugation for 3 min at 1,000 × *g* in a sucrose gradient, as described earlier (18). After centrifugation, the cells formed a visible band about 1 to 1.5 cm in width. Approximately 0.1 ml of the cell suspension was removed from the top of the band and reinoculated into 10 to 12 ml of the filtrate, which was maintained at 37°C in an Erlenmeyer flask (125 ml) in a shaker water bath to provide the synchronous culture. These cells were immediately examined for cell size and volume in a Coulter counter. The experiment was aborted if the mean volume of the selected cells was greater than about one-half the mean cell size in the exponential culture or if the maximum selected cell volume was greater than the population mean.

Buoyant density. Cells from exponentially growing cultures were fixed by the addition of 0.37% formaldehyde and then separated by size on a sucrose gradient for subsequent determination of buoyant densities by the method of equilibrium centrifugation in Percoll gradients prepared in the growth medium (17). The buoyant density of cells in synchronously growing cultures was also determined in Percoll gradients. Samples (0.5 ml) of cells were removed at intervals from synchronous cultures, sonicated (Branson, B-12, 80 W) for 15 s to disperse the cells, and fixed by the addition of 0.37% formaldehyde. Approximately half of each sample was used to determine the cell volume distribution, and half was layered on a Percoll gradient and centrifuged at 19 to 21°C for 10 min at 23,000 × *g*. Fixation was necessary to prevent cell growth during centrifugation or during storage of samples collected during the synchronous culture (30 to 40 min). Comparison of cells before and after exposure to 0.37% formaldehyde showed that buoyant densities were increased slightly (0.46 ± 0.10% [standard error, SE] by fixation. Cell volumes were also increased by fixation (0.92 ± 0.40% SE). All densities and volumes were corrected to those for unfixed cells.

When the cell concentration was sufficiently large, a visible band with a width of approximately 1 to 2 mm formed during centrifugation. To determine buoyant cell densities,

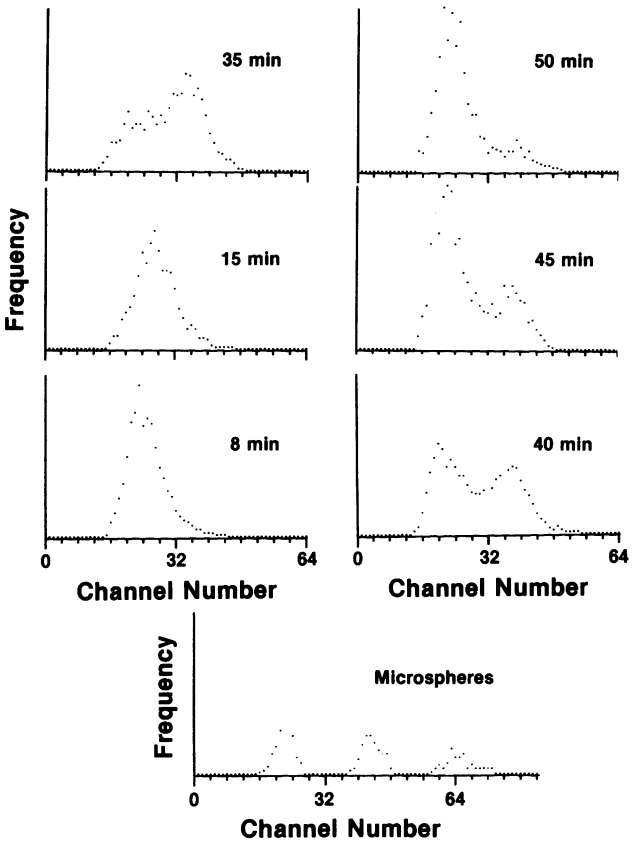

FIG. 1. Cell volume distributions in a synchronous culture of *E. coli* B/rA. Observed frequencies are shown as a function of channel number (relative cell volume) at several times during synchronous growth. The distribution of cell volumes of singlet, doublet, and triplet microspheres of 1.13-μm diameter provides a calibration of the volume scale and the resolution of the system.

75-μl samples were extracted from the top and the bottom of the band, and the refractive index of each was measured with an Abbe refractometer (17).

Sometimes cell densities in the synchronous cultures were too low to produce visible bands. The requirement for a sufficiently large number of cells for band visibility is in conflict with the requirement for selection of a small fraction of the cells for good volume resolution, and these opposing requirements provided only a very narrow range of operation. When bands were not visible, a second, less accurate procedure was used. Samples (100 μl) were removed at 2-mm intervals down the gradient, and 20 μl of these samples was used to estimate the relative concentrations of bacteria under a phase microscope. Indices of refraction were determined from the remaining 80 μl of each of the two or three samples containing the maximum cell concentrations. The errors in the buoyant density values obtained in this manner were two to three times as large as those for visible bands.

Cell counts and volumes. Cell counts and volume distributions were determined with a modified Coulter counter multichannel analyzer system (18), with a sensing aperture 16 μm in diameter. Samples were counted and sized in 0.1 N HCl. Size distributions were collected, and the data were stored with an Apple II+ microcomputer. The apparatus was calibrated for cell volume with standard latex microspheres, 1.13-μm diameter. The instrumental response was proportional to the volume (channel number) of the particles passing simultaneously through the aperture (Fig. 1). The sharpness of the individual peaks of this calibration indicates the resolution of the instrumentation: the coefficient of variation for single microspheres was 4.5%.

Figure 1 also presents the cell size distributions observed for a synchronous culture. Initially there was a single peak with increasing volume as cells grew without division. Later (35 min), a second, smaller peak appeared as some of the cells divided to give daughter cells. The amplitude of this peak increased with continued division (40, 45, and 50 min) and that of the larger peak of undivided cells decreased. Mean cell volumes were determined from the distributions for undivided cells. For increased precision in fitting this peak, the data were first smoothed by calculating a running average over every five adjacent channels, and then they were fitted with normal distributions to each peak. The cumulative normal distributions were truncated at amplitudes of about 3 and 97% to reduce the effects of noise and small tails of the distribution.

Cell growth parameters. In exponentially growing cultures, the doubling time (T) was calculated for each experiment from regressions fitted to the logarithm of the observed values for increasing turbidity, mean cell volume ($\overline{V}$) was determined from observed cell volume distributions, and mean cell mass (M) was determined as the product of the mean cell volume and the observed mean buoyant density (ρ).

In synchronous cultures, doubling times were determined from increases in both mean cell volume and cell number. In the first case, the mean cell volume at birth (V_b) was assumed to be the same for both exponential and synchronous cultures, and the doubling time was calculated as the period required to double this cell volume. In the second case, the doubling period was calculated as the interdivision period between cell birth and the time of the first synchronous cell division. If cell growth is unperturbed by the synchronization procedures, then these values are equal and also the same as the doubling time in the parent culture.

When cell growth is unperturbed in a synchronous culture, the birth volume (V_b) is unchanged and the mean cell volume during a division cycle also follows the relationship $V_b = \overline{V} \ln 2$ (18). The mean volume of the cells initially selected for synchrony was almost invariably greater than V_b because cell growth continued during the separation process, so the beginning of the synchronous cycle was identified as the time at which cell volume would have been equal to V_b from the regression fitted to the data for volume increase. Having established the beginning of the cycle, the cell division period for increase in cell number was calculated as the elapsed time for cell numbers to increase by the factor $\sqrt{2}$, the logarithmic mean for number doubling. The cell division period for increase in cell volume was determined as the elapsed time for mean cell volume to double. Estimates of the total increase in volume during the cycle were obtained from the observed rate of increase in cell volume multiplied by the length of the interdivision period as determined by the increase in cell number.

RESULTS

Cell buoyant density. Figure 2 shows cell buoyant densities determined as a function of mean cell age ($\overline{V}/V_b$) in an exponentially growing culture of *E. coli* B/rA in MSGT medium. Within experimental errors, buoyant density was independent of cell age, with an average value (± SE) of 1.0938 ± 0.0007 g/ml.

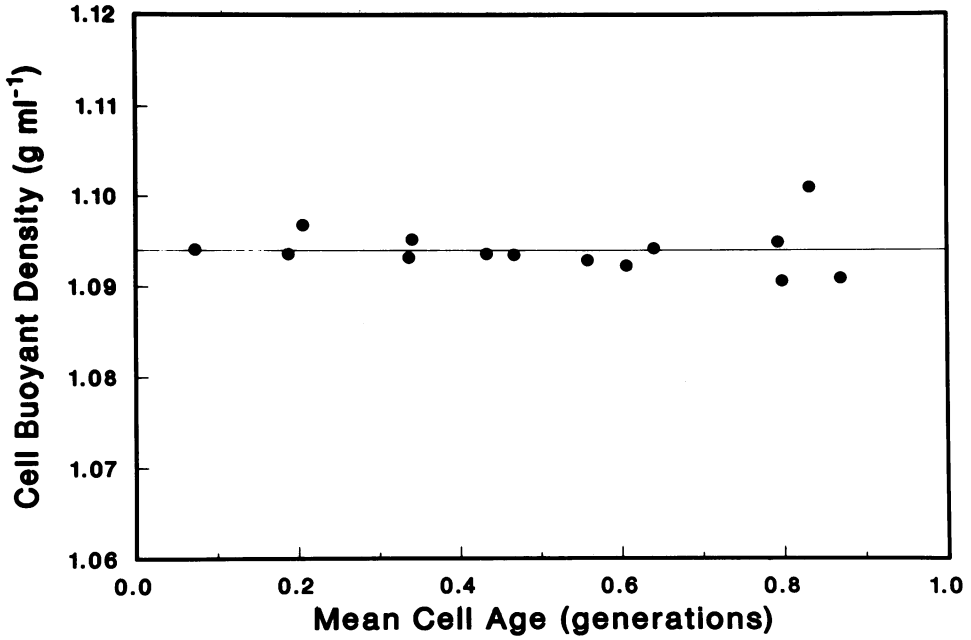

FIG. 2. Cell mean buoyant densities during the division cycle of *E. coli* B/rA in MSGT medium.

Cell growth. Mean cell volumes increased linearly during the cell cycle in every experiment (data not shown), in agreement with earlier observations (13). The cell volume data for all the experiments are collected in Fig. 3A, which shows values of relative mean cell volume (V/V_b) of the undivided cell fractions as a function of mean cell age (a). The latter was calculated from $a = (t - t_0)/T$, where t is the time that the sample was taken, t_0 is the time at which the cell volume was V_b, and T was the time observed for the doubling of the mean cell volume. The data in this figure are for nine experiments in MSGT medium, with a mean doubling time of about 40 min, and three experiments in MSGT-CAA medium, with a mean doubling time of about 25 min. The figure also shows theoretical curves for three growth models: linear, exponential, and bilinear growth with rate doubling.

The model of linear growth fits the data best, whereas the exponential growth model predicts values below almost all of the data points (Fig. 3A). Numerical values for the goodness of fit of the two models are given in Table 1. The agreement with the linear growth model is excellent, whereas the probability that the exponential model fits the data is negligible.

It is evident that most bilinear growth models are also ruled out because the points of rate doubling are well below even those for exponential growth. However, the experimental results do not rule out bilinear growth with rate doubling immediately before or after cell division. A quantitative estimate of the range of permissible values for rate doubling was determined from the values of the slopes of the regression for linear growth, for which the probability is 0.05, by considering these as permissible slopes for the first or second segment of bilinear growth models. When this kind of quantitative estimate was made, the only acceptable bilinear growth models were those that had rate doubling points at cell ages within 0.03 generations of cell division. (Details of the calculation will be furnished on request.)

The corresponding data for cell buoyant densities in these synchronous cultures are shown in Fig. 3B. These values include both the divided and undivided fractions. Although these data are necessarily more variable than those in Fig. 2, they provide evidence that the mean buoyant densities of these cells were essentially constant during synchronous growth, as the slopes of the regressions to those data did not differ significantly from zero (Table 2). Furthermore, the mean values of these buoyant densities did not differ significantly from that determined as a function of cell age, as shown in Fig. 2. The constancy of these densities in the two kinds of experiments support the conclusion that growth was essentially unperturbed in the synchronous cultures.

Further evidence for the absence of perturbation of cell growth in these experiments is given in Table 3, which compares doubling times, buoyant densities, cell volumes, and cell mass in the exponentially and synchronously growing cultures. Within statistical errors, the corresponding values were the same in all cases.

An important question concerns the effect of changes in cell shape on measurements of cell volume with the Coulter counter, as the cells are more spherical at birth than later in the cell cycle. Trueba and Woldringh (27) measured cell lengths and widths in populations of *E. coli* B/rA by electron micrography at two doubling times, 22.5 and 60 min. The aspect ratio (cell width/cell length) increased from approximately 2 to 4 from birth to division. Kachel (8) calculated the effect of cell shape on pulse height for prolate ellipsoids of various aspect ratios, and if this shape is descriptive for *E. coli* B/rA, pulse heights are too large by about 21% at birth and about 7.5% at division. Although cells of *E. coli* are probably blunter than ellipsoids, I used Kachel's values to correct the observed cell volumes and tested the various models for their goodness of fit to the shape-corrected data. The results (data not shown) demonstrated that the correction for cell shape did not influence significance levels or the choice of the growth model: the results again supported linear cell volume increase, while exponential and bilinear growth models were untenable.

Finally, because buoyant densities were constant while mean cell volumes increased linearly during the cell cycle, it

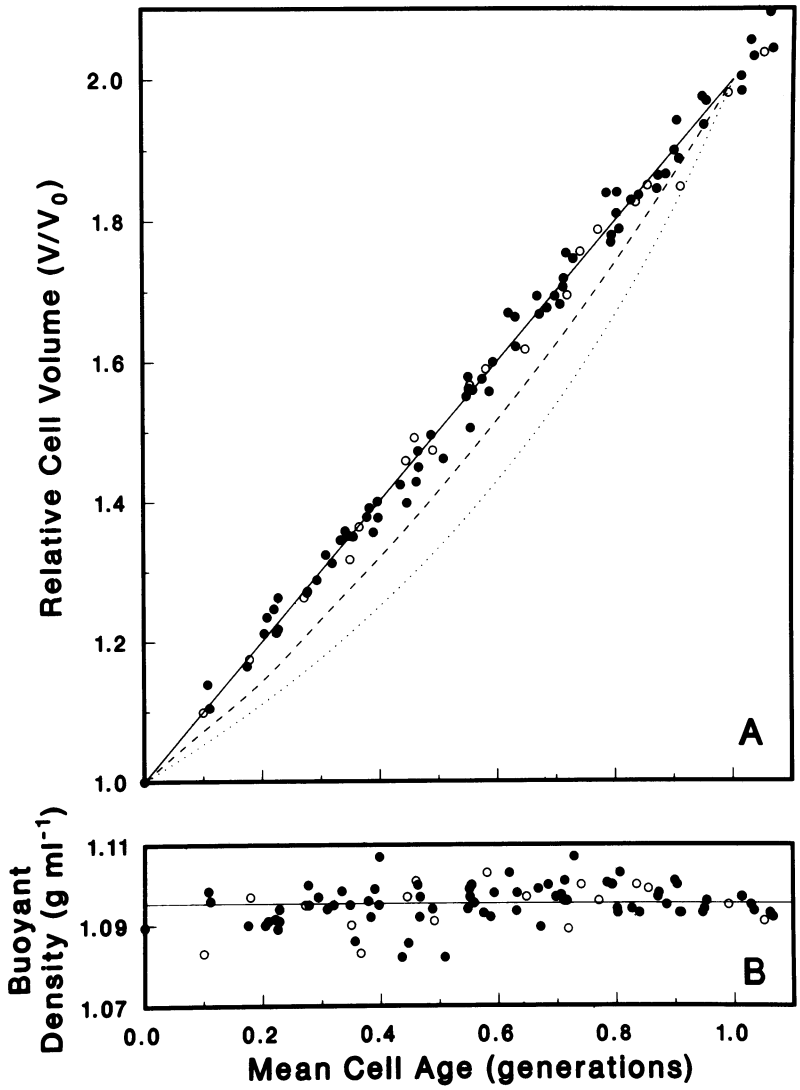

FIG. 3. Relative cell volumes and buoyant densities as a function of cell age for *E. coli* B/rA. Symbols: ●, growth in MSGT medium; ○, growth in MSGT-CAA medium. (A) Increase in cell volume in synchronous cultures. (B) Corresponding cell buoyant densities for the synchronous cultures. The straight line is the increase expected for linear growth with doubling in cell mass at one generation. The dashed line is the increase expected for exponential growth. The dotted line is the locus of rate-doubling points for bilinear growth. The bilinear increase associated with each point on this curve was obtained by extending line segments to the values of 0 and 1 generations.

is concluded that cell mass also increased linearly during the division cycle. The same conclusion was obtained when cell mass for each data point was determined as the product of the mean cell volume and the cell buoyant density for that point (data not shown).

DISCUSSION

The results presented here provide the first determinations of the patterns of increase in cell volume and buoyant density in the same bacterium and therefore the first deter-

TABLE 1. Significance tests of regression parameters for cell growth

Growth model	Doubling time (min)	Slope ± SE	P_1[a]	Intercept ± SE	P_2[b]
Linear	40	1.002 ± 0.010	0.86	0.999 ± 0.006	0.87
	25	0.998 ± 0.021	0.91	0.992 ± 0.014	0.58
Exponential[c]	40	0.918 ± 0.028	<0.01	1.100 ± 0.018	<<0.01
	25	0.928 ± 0.012	<<0.01	1.099 ± 0.008	<<0.01

[a] Probability associated with the slope statistic.
[b] Probability associated with the slope intercept.
[c] Value for observed cell volume V transformed to $1 + (\ln V/V_0)/\ln 2$.

TABLE 2. Mean buoyant densities of *E. coli* B/rA cells in synchronous cultures

Doubling time (min)	$\rho^a \pm$ SE (g/ml)	Slope$^b \pm$ SE (g/ml per h)	P_r^c
40	1.0953 ± 0.0006	0.0042 ± 0.0022	0.06
25	1.0947 ± 0.0013	0.0061 ± 0.0049	0.23

[a] Mean buoyant density.
[b] Slope of linear regression of buoyant density versus divisions per hour.
[c] Probability associated with zero slope.

mination of increase in bacterial cell mass during the division cycle. The quantitative and qualitative variation in the kinetics of growth of the individual cells in Mitchison's (21) earlier attempts to measure cell mass increase in individual cells of *Streptococcus faecalis* by optical interference microscopy did not permit the conclusion that a single, consistent growth pattern existed. In contrast, my results provide clear evidence that increase in cell mass was linear or extremely close to linear during the division cycle at both doubling times, 40 and 25 min.

The kinetics of increase in cell mass with age (Fig. 3) essentially rule out an exponential increase for *E. coli* B/rA under the standard growth conditions that I used for this bacterium. The experimental results also contrast strongly with the bilinear pattern of cell growth that I suggested earlier from an analysis of electron micrograph data (16). However, the cells were flattened and deformed in cross section during specimen preparation (30), and the disparity between the growth patterns would seem to indicate that the fixation technique requires further examination. The results in Fig. 3 also essentially rule out a bilinear increase in cell mass during the division cycle unless the age at rate doubling is within 3% of that of cell birth or division.

Although there have been many other studies of growth in *E. coli*, they have provided no basis for a consensus for cell growth kinetics. In the earliest studies, cell length was measured by optical microscopy, but optical measurements of these small cells, approximately 0.5 μm wide by 1 to 2 μm long, were limited by optical resolution, ~0.4 to 0.5 μm. This limitation undoubedly accounts in part for the very different patterns of cell length increase reported in studies that used this optical technique with *E. coli* B/r: namely, exponential (25), bilinear (2), and even interrupted growth (7).

The results presented here are also in contrast to those of Harvey and his colleagues (5, 6) obtained for *E. coli* B/r with the Coulter counter. They measured cell volume distributions in exponentially growing cultures, but their results clearly suffered from faulty instrumental resolution (15; their Fig. 2) due to a manufacturer's artifact in preparation of the sensing aperture (12, 15). Furthermore, as Sargent (24) noted, the observation of an extremely broad range of growth rates and of decreased growth rates in the largest cells were in marked contrast to the results obtained in many studies of individual bacteria.

Other, more recent determinations of synchronous cell growth with the Coulter counter have assumed that growth is represented by the increase in average, modal, or total cell volume throughout the cycle (20, 26). Conclusions become incorrect as soon as some cells divide. Divided cells have advanced to the next generation, and their ages and volumes differ greatly from those of the undivided cells and therefore cannot be averaged together with the undivided cohort. As more and more of the cells divide, the average age of the cells in the synchronous culture decreases with time near the end of the division period rather than increases, as assumed. Clearly, the measure of cell volume increase during the synchronous cycle is that for the undivided cell fraction; for these cells, age and volume increase throughout the unperturbed synchronous cycle in the same fashion as in exponentially growing cultures.

Increase in cell length or volume has also been equated to growth, but these measurements alone cannot describe the increase of the fundamental growth parameter, cell mass, during the cycle. For example, it was assumed that cell diameters of the rod-shaped *E. coli* bacteria are constant during the division cycle and therefore that cell length is closely proportional to volume and increases in essentially the same fashion (25), but in fact the only evidence (with sufficient resolution), obtained by electron micrography, shows that the mean diameter of *E. coli* cells decreases through midcycle (27). In addition, cell volume itself cannot be proportional to mass during the division cycle unless cell buoyant density remains constant. Thus, one cannot expect, in general, that cell length or volume need increase with the same kinetics as cell mass.

The results reported here for linear growth of *E. coli* B/rA satisfy the several criteria discussed above: (i) investigation of the fundamental growth parameter, (ii) absence of significant perturbation of cell growth, and (iii) achievement of the necessary instrumental and experimental resolution. Cell growth kinetics of this kind provide information on the operation of the major regulatory systems for cell growth, including the timing and magnitude of cell growth processes

TABLE 3. Mean values of parameters for cells during exponential and synchronous growth in two minimal salts media

Medium and growth (no. of expts)	Doubling time (min)[a]	Density (g/ml)	Volume (μm^3)[b]	Mass (pg)[c]
MSGT (9)				
Exponential	39.9 ± 0.4	1.094 ± 0.001	1.029 ± 0.010	1.126 ± 0.015
Synchronous	41.8 ± 3.6 (40.2 ± 3.2)	1.095 ± 0.001	1.013 ± 0.060	1.109 ± 0.060
MSGT-CAA (3)				
Exponential	25.0 ± 1.1	1.093 ± 0.003	1.517 ± 0.059	1.658 ± 0.059
Synchronous	26.1 ± 2.5 (24.0 ± 0.6)	1.095 ± 0.001	1.566 ± 0.114	1.714 ± 0.114

[a] Time required to double the mean birth volume. Numbers in parentheses are doubling times determined from the increase in cell number for the period between the time of mean birth volume and mean time of division.
[b] From rate of cell volume increase multiplied by the number-doubling time.
[c] Mean cell volume multiplied by the buoyant density.

and the nature of the biosynthetic systems that may be involved, and eliminates those that cannot conform to the observed kinetics. For example, exponential cell growth would indicate a continuously expanding capacity for uptake of nutrients, as well as for metabolic synthesis during the cell cycle. Linear growth, on the other hand, requires that the rate of cell mass increase be constant during the cycle, and this constancy in turn requires that net transport of materials into the cell also be constant. It appears, therefore, that the major growth-limiting regulatory systems operating during the steady-state growth cycle are those concerned with the passage of materials into the cell (14). Macromolecular synthesis has its own regulatory systems, of course, but these systems do not appear to be directly responsible for regulation of cell growth during the cycle.

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