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Autoradiographic Studies of Chromosome Replication during the Cell Cycle of *Streptococcus faecium*

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Analysis of the distribution of autoradiographic grains around cells of *Streptococcus faecium* which had been either continuously or pulse-labeled with tritiated thymidine (mass doubling time, 90 min) showed a non-Poisson distribution even when the distribution of cell sizes in the populations studied was taken into account. These non-Poisson distributions of grains were assumed to reflect the discontinuous nature of chromosome replication. To study this discontinuous process further, we fitted an equation to the grain distribution observed for the pulse-labeled cells that assumed that in any population of cells there were subpopulations in which there were zero, one, or two replicating chromosomes. This analysis predicted an average time for chromosome replication and for the period between completion of rounds of chromosome replication and division of 55 and 43 min, respectively, which were in excellent agreement with estimates made by other techniques. The present investigation extended past studies in indicating that the initiation and completion of rounds of chromosome replication are poorly phased with increases in cell volume and that the amount of chromosome replication may be different in different cell halves.

Recently we showed by whole-cell autoradiography that exponential-phase cells of *Streptococcus faecium* increased in RNA and protein content as they increased in cell volume and that there appeared to be no significant discrepancies during the cell cycle between cell volume and cytoplasmic mass (14a). These conclusions were based on an analysis in which the distribution of autoradiographic grains around cells was shown to fit a Poisson distribution adjusted to reflect the distribution of cell volumes found in the same population of cells used for autoradiographic analysis (the volume-adjusted Poisson). The rationale was that if cells were all of the same size, a normal Poisson distribution should fit the distribution of grains because the cells should contain the same amount of RNA and protein and hence of radioactivity. But cells change in size, protein, and RNA content and the rate at which these components are synthesized during the cell cycle; thus, the Poisson distribution does not describe the entire population and must be adjusted by weighting the Poissonian contribution of the cells in each size class by the numbers observed in the actual population studied.

We have applied this analysis to cells which were allowed to incorporate radioactive precursors of DNA over short and long labeling periods. The volume-adjusted Poissons did not give a good fit to the grain distributions for either continuously or pulse-labeled cells. This is no doubt due to the presumed linear and clearly discontinuous nature of chromosome replication (12, 15, 16). However, by fitting the sums of several Poisson distributions, each representing a discrete level of DNA synthesis, we were able to achieve a more satisfactory fit and to gather information about the phasing of chromosome replication in the cell cycle of this organism. This fitting procedure assumed that chromosome replication occurred in various subpopulations of cells in which there were zero, one, or two replicating chromosomes, each functioning at the same constant rate.

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MATERIALS AND METHODS

Cell growth. Cells of *S. faecium* ATCC 9790 were grown at 37°C in a chemically defined medium as described previously (23). The mass doubling time of cultures was ca. 90 min and was obtained by reducing the glutamic acid concentration of the medium to 20 μg/ml in the absence of glutamine (27).

Before being used for study, cultures were required to undergo 12 doublings in mass (22). For continuous-labeling conditions, high-specific-activity [*methyl*-^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) was added to a final concentration of 20 μCi/ml in a medium which contained 15 μg of unlabeled thymidine per ml. For the pulse-labeling experiments, [*methyl*-^{3}H]thymidine was added to a final concentration of 20 μCi/ml and 0.12 μg/ml, without carrier. For pulse-labeling experiments, incorporation was stopped by the addition of formaldehyde (8% final concentration), and unlabeled thymidine was added at a concentration that was 1,000 times greater than that of radioactive thymidine. Under both pulse- and continuous-labeling conditions, >99% of cold trichloroacetic acid (TCA)-precipitable radioactivity was solubilized by DNase. The flow of radioactive precursors into plasmids or repair synthesis was considered a minor fraction of the total incorporated label on the basis that (i) only about 1 to 2% of the incorporated radioactivity was found in a resident plasmid peak when cell lysates were fractionated on ethidium bromide-cesium chloride gradients (5) and (ii) in the presence of 50 μM 6-(*p*-hydroxyphenylzao)-2,4-dihydroxypyrimidine (HPAU), cells pulse-labeled for 5 min in the presence of 40 μCi of radioactive thymidine per ml incorporated <1.5% of the label incorporated by untreated controls. HPAU is a potent inhibitor of chromosome replication in *S. faecium*, and this inhibition is not associated with increased degradation of DNA to TCA-soluble form (2). HPAU has been shown to block synthesis by DNA polymerase III in *Bacillus subtilis*, but not DNA replication of one of its virulent phages (1, 6).

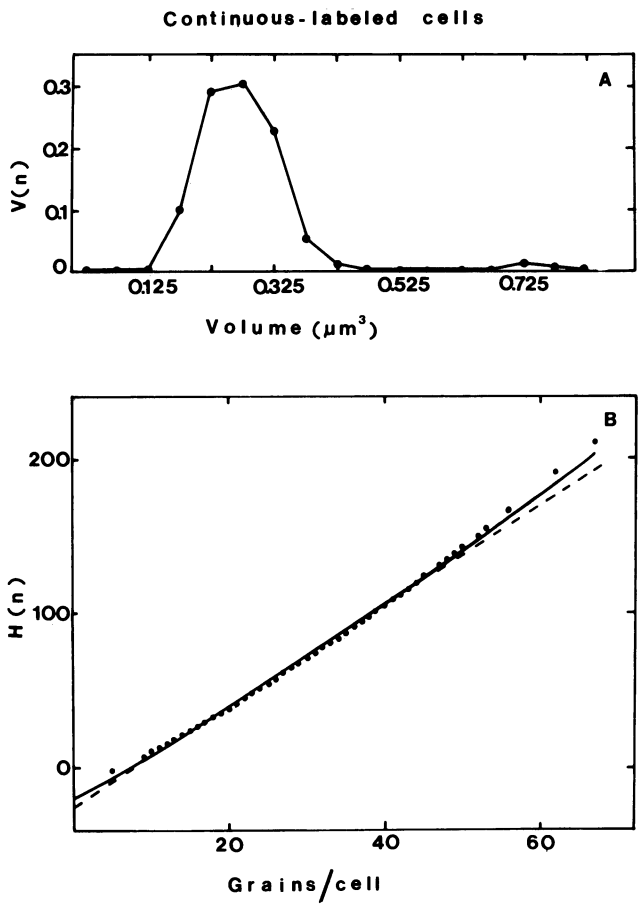

FIG. 1. Analysis of autoradiograms of whole cells of *S. faecium* which had been continuously labeled with tritiated thymidine for 12 generations before being fixed. (A) Observed frequency distribution of cell volumes [$V(n)$]. (B) Points are the frequencies of cells showing a given number of autoradiographic grains, transformed by the equation $H(n) = 1n[P(n) \cdot n!]$, where $P(n)$ is the fraction of the population with a given number of grains (n). The dashed line is a normal Poisson distribution produced by using the equation $P(n) = m^n \cdot e^{-m}/n!$, where n is the number of grains around each cell and m is the mean number of grains per cell. The solid line is a volume-adjusted Poisson (equation 2) in which the Poisson distribution has been corrected on the assumption that cell volume as seen in panel A is directly proportional to tritium content.

Electron microscopy. The electron microscopy procedure has been described previously (14a). Briefly, cells were fixed in formaldehyde, critical-point dried on carbon-stabilized Formvar films, carbon coated, coated with undiluted Nuclear Research Emulsion L4 (Ilford, Ltd., Basildon, Essex, England), and incubated in the dark. The emulsion was developed by the gold latensification procedure (21). Autoradiograms were photographed with a Hitachi-H600 electron microscope at an instrumental magnification of 20,000× with a mixture of secondary and transmitted electrons. The cells observed in micrographs were reconstructed by computer-assisted, three-dimensional imaging techniques to estimate cell volume (7, 10, 13).

Volume-adjusted Poisson distributions. The calculation of volume-adjusted Poissons has been described elsewhere (14a). Briefly, the normal Poisson equation was used for each cell size class on the assumption that the distribution of grains around cells with radioactivity was proportional to cell volume. The equation used was $P_i(n) = (F \cdot V_i)^n \cdot e^{-F \cdot V_i}/n!$ (eqn. 1), where V_i is the average volume of each size class of cells, F is an adjustable parameter used in fitting the distribution produced by this equation to an actual set of data points, n is the number of grains around a cell, and $P_i(n)$ is the fraction of the population of cells of size V_i predicted to have that number of grains.

Fitting observed autoradiographic data to the sum of cell classes differing in discrete levels of radioactivity. We used the technique of Koch (17) as expanded by Koppes et al. (19) to analyze the fraction of cells in various size classes which are undergoing various levels of chromosome replication in pulse-labeled cells. This method was based on three assumptions. The first was that a chromosome was replicated at a constant rate throughout its length. On this basis each replicating fork had the same rate of DNA synthesis and therefore the same radioactivity during a given pulse-labeling period. If each and every cell in a population were replicating a single chromosome bidirectionally, the simple Poisson distribution would describe the grain counts over the population. However, based on past studies with other organisms as well as studies of chromosome replication in *S. faecium* cells gathered from synchronously dividing cultures (12, 15, 16), it is a reasonable presumption that some cells in any population would be in a null or gap state of DNA synthesis, while some would be replicating one and others two chromosomes. Thus, the second assumption was that the mean radioactivity of each of the three cell classes was dependent solely on the number of chromosomes undergoing replication. The third assumption was that the cells that were not replicating DNA had a nonzero level of radioactivity, derived from background, repair, etc.

If we knew the proportion of the cells in each category, the problem of weighting the contribution of each would be solved, and the treatment would have been just as in the volume-adjusted Poisson, except we would have used the proportions as the weighting factors.

Without this knowledge we had to use the data themselves to fit the proportions of cells in various classes. In an attempt to solve the problem, we tried the simplest model, with zero, one, or two chromosomes being replicated in any cell. We defined $\bar{g}0$, $\bar{g}c$, and $\bar{g}2c$ as the average number of autoradiographic grains associated with a cell with no, one, or two replicating chromosomes, respectively. The equation for this is $P(n) = (1 - c - 2c) \cdot e^{-\bar{g}0} \cdot \bar{g}0^n/n! + c \cdot e^{-\bar{g}c} \cdot \bar{g}c^n/n! + 2c \cdot e^{-\bar{g}2c} \cdot \bar{g}2c^n/n!$ (eqn. 2), where $1 - c - 2c$ is the fraction of the cell cycle in the gap or null state, and c and $2c$ are the fractions of cells with one and two replication chromosomes, respectively. The computer program used then systematically varied the independent variable for $\bar{g}0$, $\bar{g}c$, c, and $2c$ in the following manner: $\bar{g}0$ from 0.5 to 3 in steps of 0.2; $\bar{g}c$ from 0.3 to 9 in steps of 0.2; c and $2c$ from 0 to 1 in steps of 0.1. For each combination of values the distribution function was calculated and compared with the actual data points shown in Fig. 3B by means of the chi-squared test (in the very low and very high volume classes the number of observations was pooled so that the number of observations analyzed was equal to or greater than five). The best fit to the four parameters $\bar{g}0$, $\bar{g}c$, c, and $2c$ was selected on the basis of the minimum chi-squared value.

The second fitting procedure altered the approach by applying equation 2 to individual volume classes, assuming that the $\bar{g}0$ and $\bar{g}c$ values for the whole population apply to each cell class. The values of $\bar{g}0$ and $\bar{g}c$ determined by the above procedure (i.e., 1.6 and 7.1 grains per cell) allowed

more precise fitting of the fraction of cells in the c and $2c$ compartments for each volume class (see Fig. 3B). In this case, equation 2 was fitted to the grain count distributions produced by cells in each size class by systematically varying values for c and $2c$ for equation 2 from 0 to 1 in steps of 0.02. The goodness of fit was again determined by the minimum chi-squared value.

RESULTS AND DISCUSSION

Exponential-phase cells with mass doubling times of ca. 90 min were allowed to incorporate tritiated thymidine for 12 generations (continuously labeled) or 9 min (pulse-labeled). Autoradiograms of both types of labeled cells were selected in a systematic and unselected manner and photographed by electron microscopy. These electron micrographs were used to determine the volume and number of autoradiographic grains associated with each cell, and the results were stored on magnetic tape.

The resulting distributions of cell volumes and grains are given in Fig. 1 and 2 for the continuously and pulse-labeled cells, respectively. An analysis of the data shown in Fig. 2 indicated that we had insufficient numbers of large pulse-labeled cells to study in the final stages of the cell cycle with any degree of certainty. To circumvent this problem we selected 187 additional cells with large volumes from the same specimen and added them to the unselected population shown in Fig. 2A. The combined volume and grain distributions for the two populations are shown in Fig. 3.

DNA content. Figures 1B, 2B, and 3B show the frequency distribution of autoradiographic grains gathered from each group of cells after they had been subjected to a Hanawalt transformation (11) (see legend to Fig. 1). With this transformation, the grain distributions shown by the solid points in Figs. 1B, 2B, and 3B should have fallen on the dashed line if these grains were distributed according to a single Poisson function. Clearly in each case, the points followed a

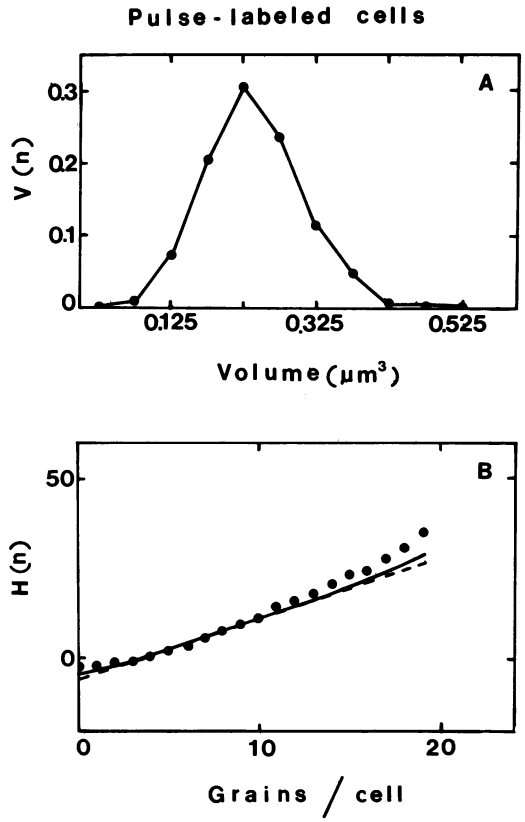

FIG. 2. Analysis of autoradiograms of whole cells of *S. faecium* which had been pulse-labeled with tritiated thymidine. Symbols are the same as for Fig. 1.

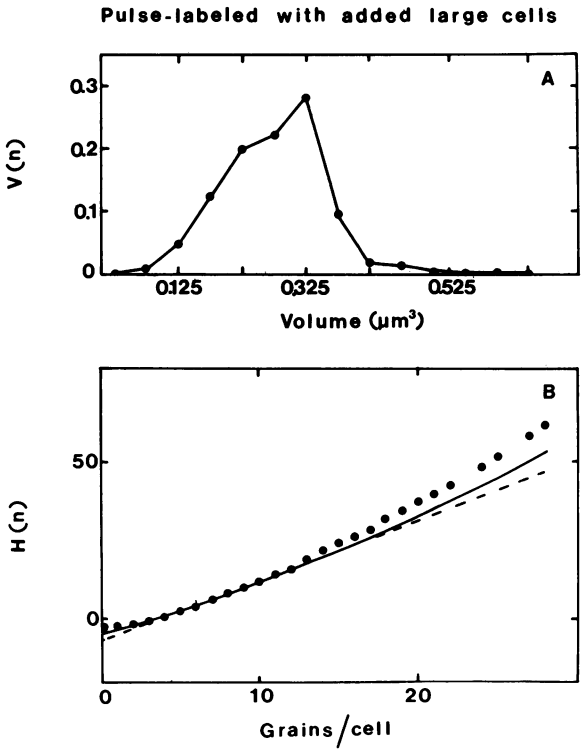

FIG. 3. Analysis of autoradiograms of whole cells of *S. faecium* which had been pulse-labeled with tritiated thymidine. To increase the precision of the analysis of cells with larger volumes, 187 large cells were selected and added to the population of cells shown in Fig. 2. Symbols are the same as for Fig. 1.

TABLE 1. Goodness of fit of normal and volume-adjusted Poisson distributions to cellular autoradiographic grain counts

Labeling condition	Type of population	D_{max}[a]		$D_{0.05}$[b]	n[c]
		Normal Poisson	Volume-adjusted Poisson		
Continuous	Unselected	0.1564	0.07442	0.0666	416
Pulse	Unselected	0.2361	0.1639	0.0642	447
	Unselected cells plus selected large cells[d]	0.2248	0.1419	0.0539	634

[a] D_{max} parameter calculated by the Kolmolgorov-Smirnov procedure. This is the maximum deviation of the cumulative experimental distribution and the fitted distribution. Normal Poisson distribution fitted to data without adjustments for the distribution of cell volumes existent in each population; Volume-adjusted Poisson distribution fitted to data which consider the distribution of cell volumes present in each population.

[b] The predicted maximum value that D_{max} may reach, and the fitted distribution that may be considered indistinguishable at the 5% confidence level from the distribution of counts established experimentally.

[c] Number of cells analyzed.

[d] Same cells used for unselected pulse-labeling determination plus 187 selected large cells.

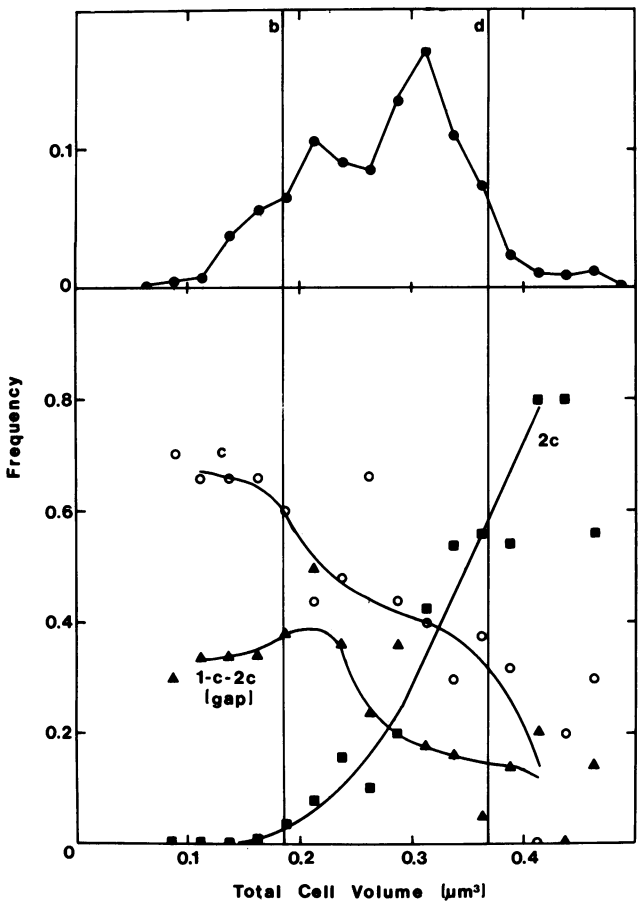

FIG. 4. Correlation between the volume distribution of cells pulse-labeled with tritiated thymidine (●, top) with the estimation of frequency of cells in each volume class (bottom), with zero ($1 - c - 2c$ [gap], ▲), one (c, ○), or two ($2c$, ■) replicating chromosomes. For the estimation of $1 - c - 2c$, c, and $2c$, see Materials and Methods. Vertical lines indicate the average volumes at birth (b) and division (d).

nonlinear, and thus non-Poisson, distribution, which indicated that the cells in each population did not contain the same amount of radioactivity. The non-Poisson nature of these grain distributions is shown in each figure by the use of the dashed line which indicated the best single Poisson function that could be fitted to the data.

The solid lines in Figs. 1B, 2B, and 3B show the volume-adjusted Poissons fitted in the present study to cells continuously and pulse-labeled with thymidine. Cells continuously or pulse-labeled with thymidine did not fit the volume-adjusted Poissons at the 5% confidence level (Table 1). This is another way of saying that two cells of the same size may not have incorporated thymidine to the same extent or at the same rate. Table 1 also shows that the D_{max} value obtained from the Kolmogorov-Smirnov test of goodness of fit (25) was considerably smaller for the continuously than for the pulse-labeled cells. Presumably these results are due to the discontinuous nature of chromosome replication within the cell cycle of bacteria (12), which is much more obvious under the pulse-labeling conditions. That the D_{max} values for the continuous-labeled cells came rather close to fitting the volume-adjusted Poisson raises the possibility that DNA replication is not strictly confined to fixed cell volume ranges during the cell cycle.

Chromosome replication. Several previous studies with light microscopic autoradiographic techniques of lower resolution than those used here have described Poisson distributions of autoradiographic grains around cells of *Escherichia coli* radioactively labeled over many generations with thymidine (3, 28); however, studies in which pulse-labeling periods have been combined with high-resolution techniques have shown that the proportion of replicating cells varies with cell length and presumably therefore with age in the cell cycle division (4). This results in a non-Poisson distribution (17, 19). It has been proposed that the shape of these non-Poisson distributions was the result of each population's being divided into subpopulations in which some cells were not synthesizing DNA, while others had one or two replicating chromosomes (19). To study the phasing of DNA synthesis in *S. faecium*, an equation for the three subpopulations was fitted to the grain distribution as described in Materials and Methods (19).

This analysis was applied to our pulse-labeled cells as shown in Fig. 4B. If the chromosome replication cycle were linked without variation to the procession of the cell through the morphological cycle, then all the cells of one size range would have one level of radioactivity and those of another size range would have a different level of radioactivity. Clearly this was not the case. Rather, as shown in Fig. 4, the fitting procedure estimated that at the average birth size (b) about 60% of the cells were replicating one chromosome (c) while the remainder were in the gap class ($1 - c - 2c$). As cells grew larger than b this procedure indicated that there was (i) an initial slight increase and then a continuous decrease in the fraction of cells in gap; (ii) an initial large decrease and then a continuous decrease of cells replicating one chromosome (c); and (iii) a continuous increase in the fraction of cells synthesizing two chromosomes ($2c$) which increased in magnitude as cells approached and exceeded the average size at division (d).

In contrast to a study in *E. coli* that used the same analysis of chromosome replication as we used (19, 20), the curves of fractions of cells of *S. faecium* in gap and replication of one chromosome (c) appeared to be more spread out and less well confined to specific portions of the cell cycle. But in both *S. faecium* and *E. coli* the regulation of chromosome replication determined by autoradiography appears to be relatively imprecise in that (i) most size classes contain sizable fractions of cells in gap and (ii) in the final stages of the cell cycle, while an increasing fraction of cells show two replicating chromosomes ($2c$), there is also a sizable quantity of cells in this same size range that have only one replicating chromosome (c).

These observations could be explained by either considerable imprecision in the phasing of chromosome replication with respect to growth and dimension of the cells or by asymmetry in chromosome replication or both. By asymmetry we mean that the two halves of the cell incorporate different amounts of thymidine due to a number of possible causes. Concerning this point in other bacteria, a recent study of *E. coli* found little evidence for large degrees of asymmetry in the initiation of chromosome synthesis at multiple origins in one cell half as opposed to the other (26); but it was shown in *B. subtilis* that there was about a 5-min difference between the termination of the left- and right-hand chromosome replication forks in a single chromosome (30).

To study the amount of asymmetry in our data, we analyzed the measurements in the manner suggested by Verwer and Nanninga (29) as extended by Koch (17). In this analysis, the number of grains in the cell half containing the

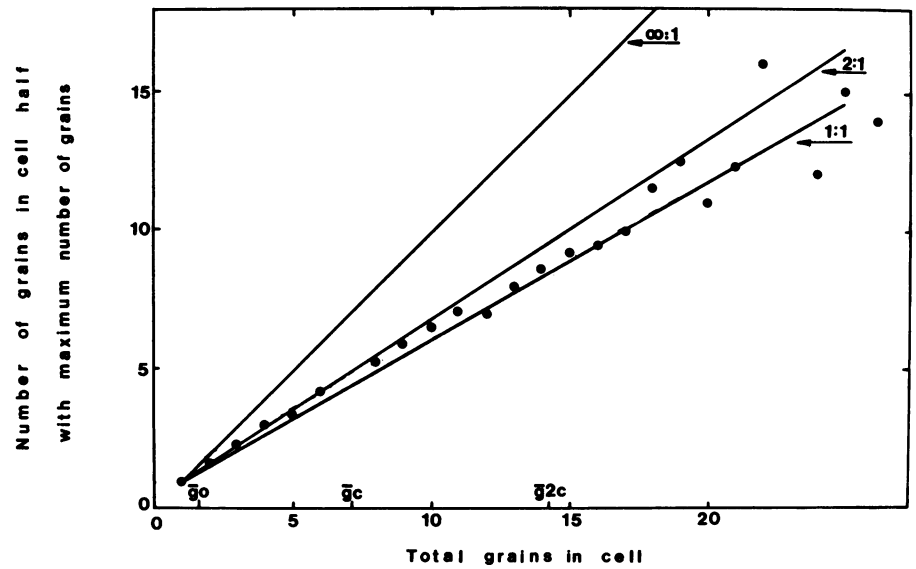

FIG. 5. Asymmetry of DNA synthesis. The number of grains on cell halves containing the highest number of grains is given on the ordinate. The theoretical predictions (21) for the case in which DNA synthesis is equiprobable in both halves of a cell is shown as the solid line marked 1:1. The case in which one of the two halves has twice the chance of having a grain is designated 2:1. Because a cell with only one grain has exactly one grain on the half with the larger number of grains, all theoretical curves and any set of experimental data will intersect the 1,1 point on the graph. Consequently the points for low grain numbers have very little error. Moreover, the experimental data for small grain counts are well represented because of the range of grain counts from DNA label, so the experimental error in the small-grain-count region excludes the 1:1 or equiprobable distribution, but the data for cells with 10 grains or more are consistent with the equiprobablity hypothesis.

largest number of grains was studied as a function of the total number of grains found per cell (Fig. 5). If one half has a greater amount of radioactivity, and therefore a different probability of forming a silver grain, than the other half, we can test this with the aid of probability theory (29). The expected average number of grains in the half with more grains for various degrees of asymmetry can be calculated and compared with the observed results. If there were no asymmetry in the distribution of grains between cell halves, the averages for each grain class would fall on the line marked 1:1. If on the contrary there were twice as much radioactivity in one half, the points in Fig. 5 marked 2:1 would apply. Finally, if the grains were distributed in a completely asymmetric manner, the points would fall in the line marked ∞:1.

As can be seen, most points fell between the lines drawn for a 1:1 and 2:1 (i.e., for grain counts from 2 to 17 for which there are a sufficient number of cells for quantitation, 12 of 15 points were above the 1:1 line). Therefore, these results were consistent with the argument that the imprecise nature of the phasing of chromosome synthesis is partly due to asymmetry. This asymmetry would be more pronounced in cells containing a small number of grains, i.e., in *c* or gap phase. The estimate of asymmetry is an underestimate because the central axis of *S. faecium* cells is rather short (ca. 1.0 μm) in relation to the average tract distance of a particle produced by tritium decay (ca. 1.0 μm). Thus, a decay on one side of the cell might produce a grain on the other side, which would make the asymmetry less distinct This would lead to much more asymmetry than indicated in Fig. 5.

This brings up the question whether some of this imprecise phasing of chromosome replication could be due to some artifact of the autoradiographic technique. To address this question we compared the average timing of the initiation and termination of rounds obtained from analyzing the autoradiographic data in Fig. 4B with times determined previously in *S. faecium* cells from measuring the fraction of cells that divided after the addition of an inhibitor or the pattern of synthesis of synchronously dividing cultures (14–16; 24; P. Borbeau, Ph.D. thesis, Temple University, Philadelphia, 1984). To analyze our autoradiograms, we again used equation 2. The cumulative percentage of cells in $1 - c$ (i.e., those cells in gap plus those in $2c$) and in $2c$ from Fig. 4B was plotted on probability paper (Fig. 6) (19). The 50% intercept in these plots indicated that, on the average, cells completed a round of chromosome replication when they reached a cell size of about 0.26 μm^3 and initiated a new round of replication at a cell size of about 0.35 μm^3. Assuming that cell volume increases exponentially during a mass doubling, these estimates then could be used to calculate that in cells with a doubling time of 90 min the average time for the termination of a round of chromosome replication during the cell cycle will occur about 47 min after birth and that a new round of chromosome replication will be initiated about 8 min before division. These times were then used to estimate that the interval between the termination of a round of chromosome replication and division (*D* time [12]) would be about 43 min (90 − 47 = 43), the average gap time in replication between rounds of synthesis would be about 35 min (90 − 47 − 8 = 35), and the time to replicate a chromosome (*C* time [12]) would be about 55 min (47 + 8 = 55). These estimates were in excellent agreement with estimates from synchronous dividing cultures and cultures measured for the number of residual divisions after the addition of an inhibitor of chromosome synthesis, which predicted *D* and *C* times for this growth rate of about 46 min (P. Bourbeau, Ph.D. thesis) and 50 to 52 min (14–16, 24), respectively.

Since the *C* and *D* times obtained by autoradiography

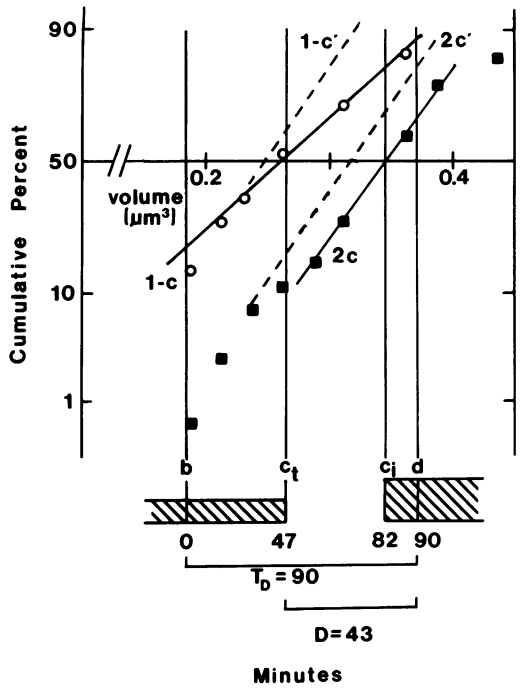

FIG. 6. Contribution of cells with one and two replicating chromosomes. The frequency data in Fig. 4 have been replotted on probability paper as a function of total cell volume to obtain cumulative frequency distributions. Only those classes with greater than 2% of the total population were included. For $1 - c$, frequencies were accumulated so that the 50% intercept would yield the average estimate of the size of cells when the first round of chromosome replication was terminated (c_i), while the frequencies of cells in $2c$ were accumulated to give the average size of cells when the second round of chromosome synthesis was initiated (c_i). b and d are the average cell sizes at birth and division, respectively, and D is the time between completion of chromosome replication and division. The cross-hatched bars indicate the average duration of chromosome replication, which in the cell cycle illustrated, coming from a culture with a mass doubling time (T_D) of 90 min, would equal about 55 min (C). Sizes were converted into estimates of time by the equation $T = (\ln(d/x)/\ln 2) \cdot T_D$, where x is the size of cells possessing an identifiable parameter and T is the average time before division that the parameter question is expressed. The dashed lines marked $1 - c'$ and $2c'$ represent the relationship between cell size and the termination and initiation of rounds of chromosome replication, assuming that a portion of cells in c actually represent the asymmetric initiation of a second round of chromosome replication in one side of the cell in advance of the other. $1 - c'$ was established by assuming that if no asymmetric initiations occurred, this line should continue to increase at the same slope as observed in small-volume classes where there would be no asymmetric initiations. The fraction of the population removed by $1 - c'$ was added to $2c$ to produce $2c'$.

were not significantly different from those deduced by more conventional methods, we conclude that the very large variations in the size of cells terminating $(1 - c)$ and initiating $(2c)$ rounds of chromosome replication (Fig. 5) are also probably valid reflections of what is occurring in living populations.

Note that the variance in size of cells terminating chromosome replication $(1 - c)$ was considerably larger than for cells initiating new rounds of replication $(2c)$ (i.e., the slope of the line for $2c$ was greater than that for $1 - c$). Possible explanations for such differences include (i) large variations in C times; (ii) reductions in the rate of growth in cell volume in subpopulations of large and small cells in which chromosome replication is not reduced to the same extent; and (iii) asymmetry in chromosome replication. Our data do not allow us to directly measure variations in C time; however, we have recently shown by autoradiography of cells pulse-labeled with leucine that there are subpopulations of very small and large cells which apparently have reduced rates of leucine incorporation (14a). In contrast, the results presented in Fig. 4 show that the fraction of cells with two replicating foci $(2c)$ increases with increasing cell size. Therefore the possibility exists that in subpopulations of very large and very small cells the normal relationship between cell size and the state of chromosome replication could be disrupted.

To assess the degree to which asymmetry could contribute to the variance between cell size and stage of chromosome replication, we hypothesized that the greater the variance in the fraction of cells terminating $(1 - c)$ and initiating $(2c)$ rounds of chromosome replication could be due to a fraction of cells that initiates new rounds of chromosome replication asymmetrically in one side of the cell in advance of the other. These cells would be scored as being in the c fraction, since only one of the two chromosomes in a cell would have initiated replication. Such a hypothesis is in agreement with the data in Fig. 5, which show that there is more asymmetry in cells which have the average number of grains associated with one rather than two replicating chromosomes (i.e., more points which have grain counts near $\bar{g}c$ fall close to the 2:1 line than those with counts near $\bar{g}2c$). The dashed lines in Fig. 6 indicate the theoretical increase in the frequency of cells terminating $(1 - c')$ and initiating $(2c')$ rounds of chromosome replication according to this hypothesis. The implications are that the actual times for the termination and initiations of rounds of chromosome replication corrected for asymmetry would be earlier in the cell cycle and lead to C and D times of 58 and 52 min, respectively (Fig. 6). However, even with this correction for asymmetry, the conclusions remain that the initiation and termination of rounds of chromosome replication appear to be spread over a large range of cell volumes and cell sizes. This is consistent with past studies of this organism (8–10, 18) in which it appeared that the regulation of chromosome replication was independent of the initiation of rounds of cell wall synthesis. Seemingly the only communication between the two processes comes at the end of the cell cycle, when cell division does not occur in the absence of termination of a round of chromosome replication.

We make the following conclusions.

(i) Autoradiographic grain distributions of cells pulse- and continuously labeled with thymidine do not fit Poisson distributions adjusted for the distribution of cell sizes in the population. This is interpreted to be due to the discontinuous nature of chromosome replication.

(ii) An equation was fitted to the grain distribution of the pulse-labeled cells which assumed that the population was made up of subpopulations of cells with zero, one, and two replicating chromosomes. According to this analysis, on the average a round of replication would terminate about 47 min into the cell cycle, and two new rounds would be initiated about 8 min before division. The interesting aspect of this study was the great variance in the size of cells in which these events occurred.

(iii) Of the various possibilities considered to account for the large variance in the size of cells terminating and initiating chromosome replication, the asymmetric initiation of chromosome replication in one side of the cell in advance

of the other appeared quite plausible based on the analysis of asymmetrical grain distributions in the population.

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