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Characterization of Cell Cycle Events during the Onset of Sporulation in *Bacillus subtilis*

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To elucidate the process of asymmetric division during sporulation of *Bacillus subtilis*, we have measured changes in cell cycle parameters during the transition from vegetative growth to sporulation. Because the propensity of *B. subtilis* to grow in chains of cells precludes the use of automated cell-scanning devices, we have developed a fluorescence microscopic method for analyzing cell cycle parameters in individual cells. From the results obtained, and measurements of DNA replication fork elongation rates and the escape time of sporulation from the inhibition of DNA replication, we have derived a detailed time scale for the early morphological events of sporulation which is mainly consistent with the cell cycle changes expected following nutritional downshift. The previously postulated sensitive stage in the DNA replication cycle, beyond which the cell is unable to sporulate without a new cell cycle, could represent a point in the division cycle at which the starved cell cannot avoid attaining the initiation mass for DNA replication and thus embarking on another round of the cell cycle. The final cell cycle event, formation of the asymmetric spore septum, occurs at about the time in the cell cycle at which the uninduced cell would have divided centrally, in keeping with the view that spore septation is a modified version of vegetative division.

Under starvation conditions, *Bacillus subtilis* undergoes a differentiation process that culminates with the release of a dormant endospore. The first overt morphological event in this process is the formation of a polar division septum positioned quite differently from the centrally located septum of the vegetative cell. Asymmetric septation generates two compartments of unequal size, a large one, the mother cell, and a smaller one, the prespore. The morphology of cells during the asymmetric division process (20, 21) and the common requirements for cell division genes, such as *ftsZ* (4), *divIC* (27), and *divIB* (3), suggest that spore septum formation is a modified form of vegetative division.

Although a great deal is known about the nature of the nutritional signals controlling the switch from vegetative growth to sporulation (22) and about the changes in transcription that accompany and direct the differentiation of prespore and mother cell (15), little is known about how the cell cycle is modified at the onset of sporulation. The starting material for sporulation is apparently a cell containing two fully replicated chromosomes, one destined for the spore and the other remaining in the mother cell (2, 19, 37, 41, 46). Consequently, when sporulation is initiated after a period of relatively rapid growth and thus dichotomous replication, the cell must undergo a complex transition to a state in which two completed, nonreplicating chromosomes are present. In order for this to occur, it seems likely that ongoing rounds of replication must be completed and new rounds avoided, but how this reorganization of the cell cycle is achieved has not been elucidated.

Two lines of evidence, obtained from experiments with populations of cells initiating sporulation asynchronously, have suggested that the onset of sporulation can occur only at a particular point in the cell cycle. Firstly, pairs of sister cells tend to be at the same developmental stage of sporulation (9), suggesting that they initiated sporulation simultaneously. This could obviously be a consequence of sister cells being synchronized with respect to the cell cycle, having been the products of the same division. Secondly, experiments with inhibitors of DNA replication suggested that cells could initiate sporulation only at a particular point in the DNA replication cycle (7, 11, 29). However, the other cell cycle parameters of cells at the postulated sensitive stage (e.g., mass and length) have not been further investigated.

The tendency of *B. subtilis* cells to grow in chains (34) precludes the use of automated cell-sorting methods to study cell cycle events in individual cells. Here we describe the development of a digital microscopic method for measuring cell cycle parameters in individual *B. subtilis* cells and its application to the study of events at the onset of sporulation. A detailed sequence of cell cycle events has been deduced for the abrupt transition from growth to sporulation by using the classical resuspension method (42). In addition to providing a picture of the changes in cell size and DNA content that occur as cells initiate sporulation, the results confirm that there is a period of sensitivity in the cell cycle during which cells can respond to a starvation stimulus and switch to asymmetric division (7, 11, 29). This point of sensitivity need not, as previously assumed, correspond to a stage within the DNA replication cycle. More likely, it represents the point at which the cell attains a size such that it cannot avoid becoming committed to another round of DNA replication, even at the reduced growth rate in starvation medium (SM). Although asymmetric septation clearly involves a modification of the vegetative septational machinery, in terms of positioning and ultrastructure, we show that its timing relative to the completion of DNA replication is similar to that of vegetative division.

MATERIALS AND METHODS

General methods. *B. subtilis* SG38 *trpC2 amyE* (16) was used for all of the experiments described. An *amyE* mutation of unknown origin was recently de-

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tected to be present in this strain (48). Strain L5481 *dnaB19*(Ts) *flaD2* (45) was used as the internal standard of DNA content in the digital image analysis. Sporulation was induced (42) by growing cells in a rich hydrolyzed casein (CH) medium and resuspending the cells in sporulation medium (SM) as specified by Partridge and Errington (33). These media were supplemented with tryptophan (20 μg/ml).

Preparation of standard cells with single completed chromosomes. Strain L5481 was used to derive the standard cells used for DNA content analysis. This strain is thermosensitive for the initiation of DNA replication and grows as chains of cells because of its deficiency in autolysins. The strain was grown in CH medium to an A_{600} of 0.45 and then shifted to 48°C for 70 min. During this incubation at the nonpermissive temperature, ongoing rounds of DNA replication are completed and more than 90% of the completed chromosomes are segregated into separate cells (45). The fluorescence of the standard nucleoids was relatively constant (the standard deviation was about 12%). A small proportion of these nucleoids (<10%) were excluded from the analysis because of their clearly higher DNA content. These may correspond to unsegregated sister chromosomes (45).

Measurement of cell length and DNA content by digital image analysis. Previously published methods for visualization of septa in *B. subtilis* (e.g., treatment with CsCl solution [13] or formalin [31]) did not allow asymmetric septa to be seen. However, we found that storage of cell samples in 70% ethanol, followed by brief rehydration, allowed both vegetative and asymmetric spore septa to be seen clearly by phase-contrast microscopy (see Fig. 1). These preparations could also be stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) and the DNA could be viewed by fluorescence microscopy, giving images similar to those reported previously for glutaraldehyde-fixed cells (6, 41).

A culture sample was chilled, and NaN_3 was added to a final concentration of 0.1% (wt/vol). The sample was microcentrifuged for 3 min (room temperature, $10{,}000 \times g$) and resuspended in 70% (vol/vol) ethanol at 4°C, after which the cells could be stored indefinitely. After at least 2 h of storage, a sample was microcentrifuged, resuspended in sterile bidistilled water to disperse the aggregates of cells formed in ethanol, and then mixed with an appropriate amount of standard cells, each containing one complete chromosome, that had been prepared in the same way. A 2- to 5-μl amount was immediately spread on a coverslip (no. 0; BDH), which had been previously treated with 0.01% polylysine (Sigma), and the suspension was allowed to dry. The coverslip was inverted onto 5 μl of 50% (vol/vol) glycerol containing 1 μg of DAPI per ml and immediately observed with a Zeiss Axiophot fluorescence microscope equipped with a 100× oil immersion objective, a 2× Optovar, a G365 exciter filter, an LP420 barrier filter, and a cooled charge-coupled device Hamamatsu C4742 camera (1,024 by 1,024 pixels). The camera was connected to a MacIntosch Quadra 900 computer through a Perceptrics frame grabber (Perceptrics, Knoxville, Tenn.). Phase-contrast and fluorescence images of an appropriate field were grabbed by using Image 1.49 processing and analysis software (written by W. Rasband, National Institutes of Health). To improve the visualization of the septa, the phase-contrast image was grabbed with a weak microscope light. (Note that although the positions of septa were very clear when viewed directly, or when reproduced photographically [see Fig. 1], they are not always visible on the digital images.) By checking directly through the microscope, the position of each septum was marked on the phase-contrast image. Because the dye fades, the fluorescence image was grabbed as quickly as possible. By using Adobe Photoshop 2.1 software, the phase-contrast image, except the marks localizing the septa, was eliminated and the image obtained was superimposed on the fluorescence one. By using the Image software, an area was drawn around each cell between the two marks indicating the positions of the septa. The length of the cell and the total density of the stain in the marked area were measured and compared with measurements of a 10-μm scale and of nucleoids of the standard cells, respectively. Aggregates of cells were ignored. Images shown in Fig. 1, 2, and 6 were printed on Ilford Delta 35-mm film. For digital images (Fig. 2 and 6), Polaroid Print-me-later 3.0.2 software was used.

Accuracy of the cell length measurements. Measurements of cell length from a single culture sample prepared for microscopy by different procedures showed that neither the ethanol fixation nor the glycerol in which the cells were stained with DAPI significantly altered this parameter (data not shown). The accuracy of length measurements was tested by measuring the lengths of spores obtained in nutrient broth. The mean length obtained by the present method (1.18 ± 0.10 μm, 38 spores) was in agreement with that obtained by traditional measurements on phase-contrast micrographs (1.12 ± 0.15 μm, 100 to 200 spores [19]).

Accuracy of DNA content estimations and compensation for effects of DNA condensation. To test the accuracy of the method for estimating DNA content, we measured the relative fluorescence of nucleoids in a single field of sporulating cells (Table 1). The four major classes of nucleoids were analyzed: those of the standard cells, those of nonsporulating cells, and those of fully condensed prespores and their associated mother cells. Two minor classes of cells, probably representing cells making the transition from vegetative growth to sporulation, were ignored for this purpose. The average fluorescence of the internal standard nucleoid, corresponding to a single complete chromosome, was set at 1. The mother cell nucleoids had an average fluorescence very close to 1, as expected if they contain single completed chromosomes (see the introduction). The prespore nucleoids exhibited a lower average fluorescence, as shown previously for *Bacillus megaterium* (41). Although it has been suggested that the lower apparent fluorescence of the prespore is due to overlapping of the out-of-focus fluorescence of the prespore and mother cell nucleoids (41), it seemed to us that the major contribution to this effect was a reduction in penetration of the excitation light with increasing DNA concentration (44). In any case, the increased condensation of the prespore DNA was evident from its maximum pixel brightness (MPB), which was about 20% greater than that of either the standard cells or the mother cells (Table 1).

TABLE 1. Relative fluorescence and maximum pixel brightness of nucleoids stained with DAPI[a]

Nucleoid type	No. of nucleoids	Avg relative fluorescence[b]	Avg maximum pixel brightness[c]
Standard	14	1.0 ± 0.12	159 ± 19
Nonsporulating cells	42	1.37 ± 0.33	199 ± 19
Prespore[d]	34	0.73 ± 0.12	206 ± 21
Mother cell[d]	34	1.01 ± 0.09	148 ± 11

[a] A single image of cells sampled 90 min after resuspension was analyzed.
[b] Relative to fluorescence of standard nucleoids. Values are means ± standard deviations.
[c] Black corresponds to a brightness of 0, and white corresponds to a brightness of 255. Values are means ± standard deviations.
[d] Prespore and mother cell nucleoids correspond to the same sporulating organisms.

The reproducibility of the measurements was illustrated by the relatively low level of variation in average fluorescence of mother cell, prespore, and standard nucleoids, which were all expected to have single completed chromosomes. The nonsporulating cells in the field showed considerable variation in fluorescence, as expected, because they were undergoing DNA synthesis and would thus be at different points in the replication cycle. The DNA of the nonsporulating cells (and of cells growing in CH medium; see below) was more condensed than that of the standard nucleoids, as indicated by the greater MPB in Table 1. We assumed, on the basis of the observations on prespore nucleoids, which seemed to be condensed to a similar level (Table 1), that this would result in an underestimation of the DNA content, and so when determining the DNA content of cells in CH medium (see below), we applied a correction factor of 1.4, based on the known underestimation of the prespore DNA. The internal consistency of the data for cells growing in CH medium justified the application of this factor (see below).

Chloramphenicol (CAM) treatment caused a high degree of nucleoid condensation (the MPB was 1.6 times greater than that of the standard nucleoids), as has been reported also for *Escherichia coli* (52). The nucleoids of the CAM-treated cells showed two levels of fluorescence. If it is assumed that these corresponded to two and four completed chromosomes, the underestimation of the DNA content caused by the condensation would be to 1.5- and 1.8-fold. This would be consistent with the effects of DNA condensation on the prespore DNA (MPB, 1.3 times the value for standard nucleoids; underestimation of DNA content, 1.4-fold [Table 1]). Other possible explanations for the two peaks (e.g., one and two nucleoids or four and eight nucleoids) are completely incompatible with the values for generation time, DNA replication time, and observed residual cell division.

The level of DNA condensation for the cells defined as being in the "preseptation" state (see Results) was slightly higher than that for the standard nucleoids (MPB, 1.1), and so the value given for their DNA content includes an estimated correction factor of 1.1.

Determination of DNA content in cultures. It appeared that the efficiency of DNA extraction from whole cells by the warm perchloric acid method (5) varied greatly from one resuspension experiment to another (18). Consequently, cells were lysed and their DNA was precipitated before being assayed by the diphenylamine method. Duplicate 1-ml samples were chilled, and NaN_3 was added (final concentration, 0.1% [wt/vol]). The samples were microcentrifuged for 3 min (room temperature, $10{,}000 \times g$), resuspended in 0.2 ml of TES buffer (0.2 M Tris [pH 7.5], 5 mM EDTA, 100 mM NaCl) containing 1 mg of lysozyme per ml and 0.05% NaN_3, and incubated at 37°C for 15 min. Sarkosyl (NL30; BDH) and pronase E (Sigma) were added to final concentrations of 1.6% (vol/vol) and 1 mg/ml, respectively, and the mixture was incubated at 37°C for 30 min. The sample was mixed with 1 volume of 2-propanol, incubated at room temperature for 15 min, and microcentrifuged for 5 min. The pellet was washed with 70% ethanol and resuspended in 0.2 ml of sterile H_2O, and the DNA concentration was measured by the diphenylamine method (5) with 2′-deoxyadenosine (Sigma) as a standard. In agreement with the findings of Mandelstam et al. (30), the DNA concentration immediately after resuspension of cells in SM was about 4 μg/ml of culture. Surprisingly, the absolute DNA concentrations found in SM were about 1.25 times lower than those in CH medium at the same time, apparently because of a lower efficiency of DNA extraction from cells in SM. However, this did not affect the conclusions drawn.

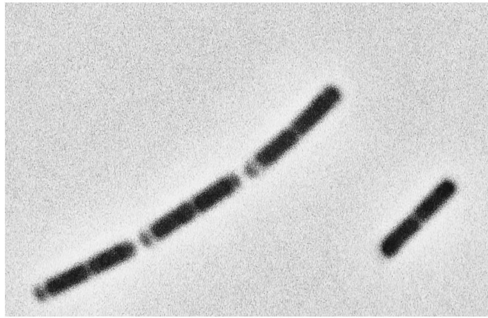

FIG. 1. Photomicrograph illustrating visualization of vegetative-stage and asymmetric septa by ethanol fixation and phase-contrast microscopy.

RESULTS

Cell cycle in rich CH medium. Of the various methods used to induce sporulation, the resuspension method (42) gives the most reproducible results, and it has the added advantage of providing a defined time point for the initiation of sporulation. It involves growth of the cells in a rich medium (CH), followed by resuspension of the cells in a starvation medium (SM). To understand how the *B. subtilis* cell cycle is reorganized during the onset of sporulation, it was necessary first to determine some basic cell cycle parameters for cells growing vegetatively in CH medium. A method for measuring cell length and DNA content in individual cells is described in detail in Materials and Methods, together with various control experiments characterizing the validity and accuracy of the method. Briefly, cell length was measured by phase-contrast microscopy of cells fixed in ethanol. Unlike previously described procedures, ethanol fixation has the advantage that it allows visualization not only of vegetative division septa but also of the much thinner asymmetric spore septa (Fig. 1). The number of nucleoids per cell and the DNA content of each nucleoid were determined by digital-imaging fluorescence microscopy of preparations stained with DAPI (Fig. 2). A culture growing exponentially in CH medium, under normal conditions used for resuspension, was analyzed by the new method. The distributions of cell

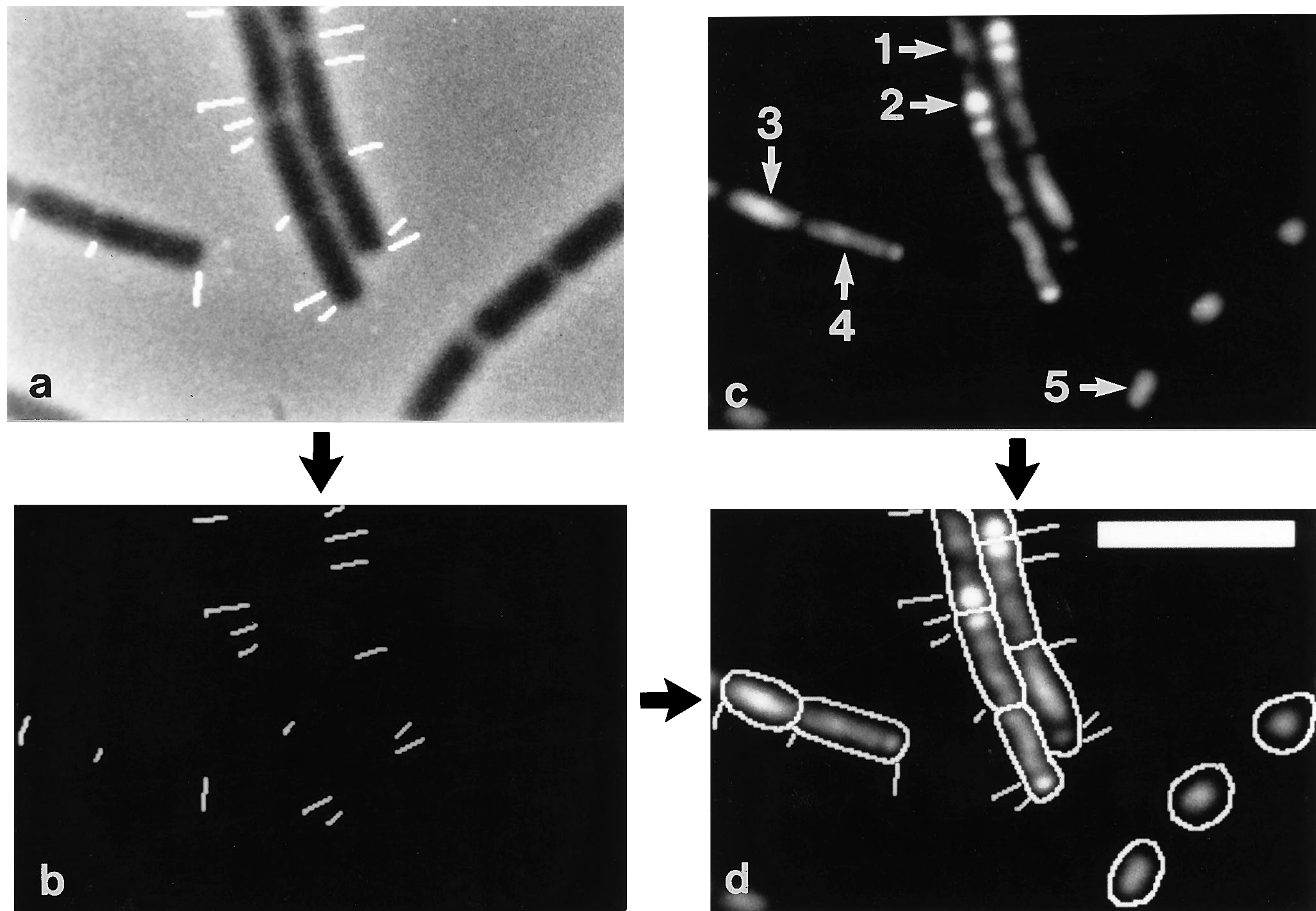

FIG. 2. Measurement of cell length and DNA content by digital image analysis. (a) The positions of septa were marked on a phase-contrast digital image. The positions of the septa were clearly visible when viewed directly through the microscope, or when reproduced photographically (Fig. 1). Marks in close proximity are due to the formation of an asymmetric septum. (b) The phase-contrast image was eliminated, leaving the marks at septal positions. (c) Fluorescence digital image of the DNA stained with DAPI, showing sporulating organisms each containing two nucleoids: the more diffuse mother cell DNA (arrow 1) and the condensed prespore DNA (2). The nucleoids of a nonsporulating cell (3), a preseptational cell (4), and a standard cell (5) are also indicated. (d) The image shown in panel b was superimposed on that shown in panel c, and a line was drawn around the periphery of each cell. Cell length and total fluorescence density in each area were measured and compared with standards. When a cell was not completely filled with DNA (for example, exponentially growing cells; see Fig. 6a), fluorescence was measured separately in an area drawn around each nucleoid. The standard cells were easily recognizable because they carried a *fla* mutation, which causes formation of very long chains of cells, and because their nucleoids had a characteristic shape and distribution. Scale bar, 5 μm. Only about 1/20 of a field is shown. The cells were sampled 75 min after resuspension.

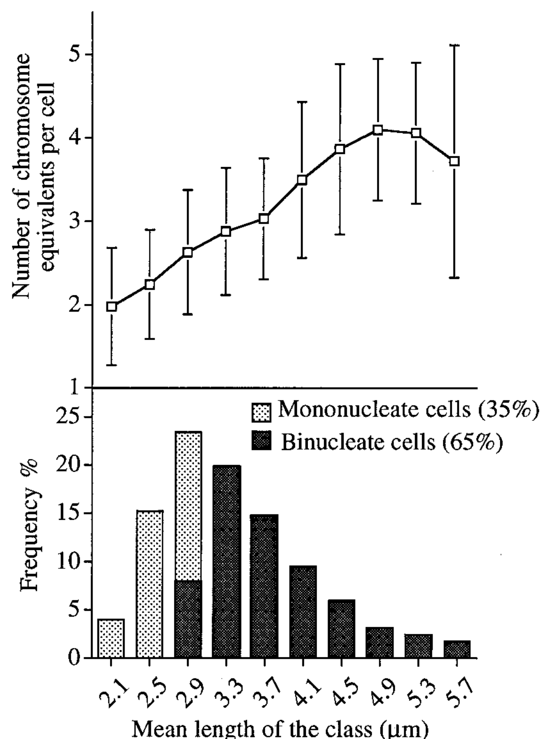

FIG. 3. Characterization of 453 cells growing exponentially in CH medium by digital image analysis. The cells were classified according to length and whether they were mononucleate or binucleate (percentage of each type shown), and the mean DNA contents and standard deviations (error bars) for each length class were calculated. The overall mean DNA content was 2.9 chromosome equivalents per cell, and the mean cell length was 3.4 μm.

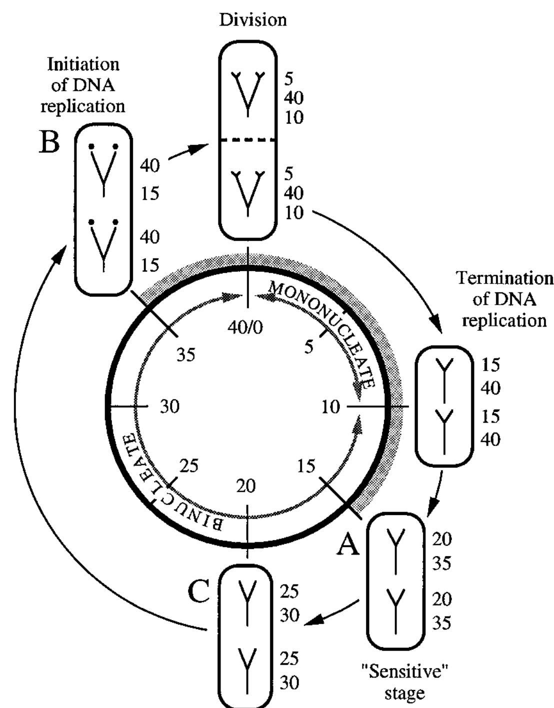

FIG. 4. Model for progress through the cell cycle in CH medium. Numbers inside the circle show time in minutes, with 40/0 representing time of division/time of birth. To simplify, the replicating circular chromosomes in each of the cells are represented as forked linear structures representing only one half of the bidirectionally replicating chromosome. The numbers by the cells represent the sizes of the replicated and unreplicated portions of the chromosome expressed as minutes of replication; the sum of the numbers is equal to the chromosome replication time of 55 min. Dots indicate the initiation of new rounds of DNA replication. Arrows inside the circle indicate the periods in the cycle when cells are predominantly mononucleate or binucleate. The sensitive stage in the cell cycle inferred from these experiments is indicated in the main sequence. The shaded semicircle indicates the period of the cell cycle in which transfer to SM could result in sporulation without the need to initiate a new round of DNA replication. A, B, and C refer to the three types of cell considered in Fig. 8 below.

length, DNA content, and number of nucleoids in the population of cells are summarized in Fig. 3. The shape of the frequency distribution, with a high proportion of small (younger) cells and a decrease in frequency with increasing size (and thus age), was, as expected, characteristic of an asynchronous exponentially growing population dividing by binary fission (25, 26). The DNA content, expressed in chromosome equivalents, increased linearly with cell length. It appeared that cells that are about to divide are, on average, binucleate (i.e., they contain two discrete nucleoids) and about 5 μm in length and contain four chromosome equivalents. Such cells give birth to two mononucleate cells of 2.5 μm, each containing two chromosome equivalents. The relatively small numbers of very long and small cells indicate that cell division is quite tightly coordinated with cell length increase, as expected.

Figure 4 shows a model for progression through the cell cycle in CH medium, based on the above results and assuming that a round of DNA replication takes 55 min (11). In the model, termination of the DNA replication round initiated during the previous cycle occurs 10 min after cell division and initiation of a new round takes place 5 min before division. The period between termination of replication and the next septation is about 30 min. The cell becomes binucleate roughly when termination of replication takes place (Fig. 3 and 4), in agreement with previous results indicating that the time between termination of replication and nucleoid partition occupies only a small portion of the cell cycle (38). The significance of the sensitive point in the cell cycle is discussed below.

To check that the model was correct and thus to confirm the validity of the cell cycle parameter measurements, we treated a similar culture with CAM. This prevents initiation of DNA replication but allows ongoing rounds to proceed to completion (50), so that at the end of a 1-h incubation, all cells should contain completed chromosomes (see reference 40 for an example of the use of this method).

The DNA contents of the nucleoids of the CAM-treated cells fell into two major peaks, which we assumed to correspond to two (30%) and four (67%) completed chromosomes (see Materials and Methods). A few cells (the remaining 3%) appeared to have eight completed chromosomes. Direct microscopic counting revealed that some cells had divided in the presence of the drug, giving an increase in cell number of about 36%, in agreement with a previous estimation (32). It seems reasonable to assume that the cells that divided were those at the latest stage in the division cycle at the time of addition of the drug. According to the model (Fig. 4), most of the cells in CH medium contain four origins of replication; only those in the last 5 min or so of the cycle contain eight. The cells that divided in the presence of CAM would thus have either four chromosomes (if they had just initiated a new round of DNA replication) or two (if they had not initiated a new round) at the time of CAM addition. The progeny of the 36% of cells that divided in the presence of CAM (see above) would constitute 53% ([36 × 2]/136) of the final population. Since the proportion of cells with only two completed chromosomes was

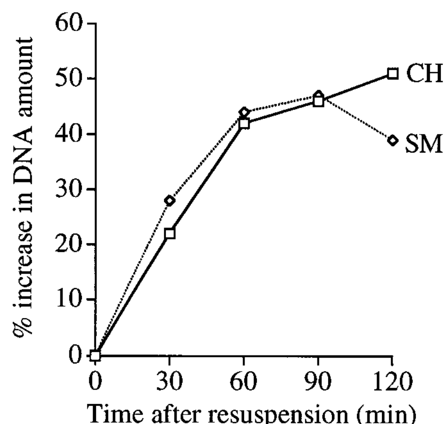

FIG. 5. Estimation of the replication rate after resuspension of cells in SM medium by the residual DNA synthesis in the presence of CAM. A culture in CH medium was divided into two equal parts, which were centrifuged. One half was resuspended in SM, and the other was resuspended in CH medium; both resuspension media were supplemented with 200 μg of CAM per ml. The DNA concentration was determined periodically, as described in Materials and Methods.

about half of this (30%; see above), the cells that divided must have fallen into two almost equal classes, at the time of CAM addition, which had either initiated or not initiated another round of DNA replication. The results thus strongly support the proposition (Fig. 4) that the initiation of DNA replication usually takes place just before cell division during growth in CH medium.

The small proportion of cells with eight entire chromosomes was unexpected, because cells with eight origins should be ready to divide. Perhaps these cells are derived from the rare unusually long cells (Fig. 3) in which cell division is apparently delayed.

Replication rate after resuspension in SM. It is widely considered that at relatively high growth rates the time required for replication of the chromosome is not affected by the richness of the growth medium; change in growth rate is compensated for by controlling the timing of initiation of DNA replication (8). Nevertheless, to understand the fate of cells following resuspension in SM, it was important to confirm that the replication rate does not change in SM. Incorporation of labelled thymidine by a thymine-requiring mutant suggested that the replication rates are similar in CH medium and SM (39, 51). However, Thy^+ strains, which could have a higher replication rate than Thy^- strains (14), have not been studied in this respect. To determine the replication rate of our Thy^+ strain in SM, we measured the residual DNA synthesis following CAM treatment, which, as mentioned above, prevents initiation of DNA replication but allows ongoing rounds to proceed to completion. A culture growing in CH medium was divided into two portions and centrifuged, and the cell pellets were resuspended either in fresh CH medium or in SM medium, both containing CAM. The subsequent increases in DNA content were then measured (Fig. 5). The rates of increase of DNA were similar in the two media, and a plateau was reached after about 60 min. The similarity of the curves obtained with SM and CH medium was consistent with there being no significant change in the rate of DNA replication after resuspension in SM. The maximum value attained with SM represented an increase of about 45%, while with CH medium there was a slow increase up to about 50%. In two other experiments, similar results were obtained except that the plateau values varied between 45 and 55% (data not shown). Both the plateau values (43) and the apparent times of completion of synthesis indicated a replication time of about 55 min. Incorporation of [^{3}H]thymidine in similarly treated cultures provided an independent means of measuring residual DNA synthesis; incorporation stopped after 60 min in both CH medium and SM (18). We can conclude that the replication time in SM is similar to that in CH medium and close to the previously estimated time of 55 min (11).

Nucleoid behavior after induction of sporulation. To determine how the cell cycle is modified at the onset of sporulation, a culture growing in CH medium and exhibiting the normal growth rate for this medium was resuspended in SM by the standard procedure (33, 42). The sporulating culture was sampled every 15 min, and cells were classified according to the appearance of their nuclear material (Fig. 2 and 6). In the early samples, the nucleoids were indistinguishable from those of the vegetative cells in CH medium and were scored accordingly as either mononucleate or binucleate. From 45 min, a new class of cell became apparent. These cells had a uniform length of 2.54 ± 0.24 μm and an estimated DNA content of 1.85 ± 0.29 chromosome equivalents (67 cells measured) (see Materials and Methods). Their DNA was less condensed, more or less filling the cytoplasm, rather than occupying a discrete spherical or ovoid region. This class apparently corresponds to an early step in sporulation because their average cell length and estimated DNA content were both just less than those of sporulating organisms (2.68 ± 0.25 μm and 2.00 ± 0.22 chromosome equivalents, respectively; 65 cells counted). Cells of this type were therefore assigned to a class called the preseptation class. The occurrence of an elongated, more diffuse nucleoid during stationary phase has been reported previously (6), and this form may be related to the axial filament seen by electron microscopy (36). The last class of cells observed were sporulating organisms, and in this class we included cells with a visible asymmetric septum or two nucleoids, one small and highly condensed (the prespore) and one larger and much less condensed (the mother cell). A proportion of the sporulating organisms with asymmetric septa did not have condensed prespore DNA. This is due to the fact that prespore DNA partitioning seems to occur by translocation through the asymmetric septum (47, 49). This subclass represented 82, 30, and 20% of the sporulating organisms at 60, 75, and 90 min, respectively. In agreement with previous estimations, comparison of the time of appearance of asymmetric septa (60 min [33]) with that of the appearance of fully condensed prespore DNA (70 min [28]) suggests that translocation of the prespore chromosome takes about 10 min.

The variations in the proportions of the different cell classes described above are shown in Fig. 7. During the first 45 min, binucleate cells became less frequent and were replaced by mononucleate cells. This was expected, because a reduction in the rate of DNA replication reinitiation, due to the nutritional downshift, should lead to a reduction in the number of replicating chromosomes per cell and thus of nucleoids. Such a reduction of the mean number of nucleoids per cell during a downshift has been described for *Salmonella typhimurium* (24). The proportion of preseptational cells increased from 45 min until 75 min and then fell, these cells apparently being replaced by sporulating organisms. The latter increased from 60 min to 120 min, reaching about 70% of the cell population in this experiment. There was little if any increase in the numbers of sporulating organisms after 120 min.

To determine the degree of synchrony of sporulation in sister cells during the early stages of sporulation, the state of development in cell pairs was examined by using samples taken 75 and 90 min after initiation of sporulation. The sister cells

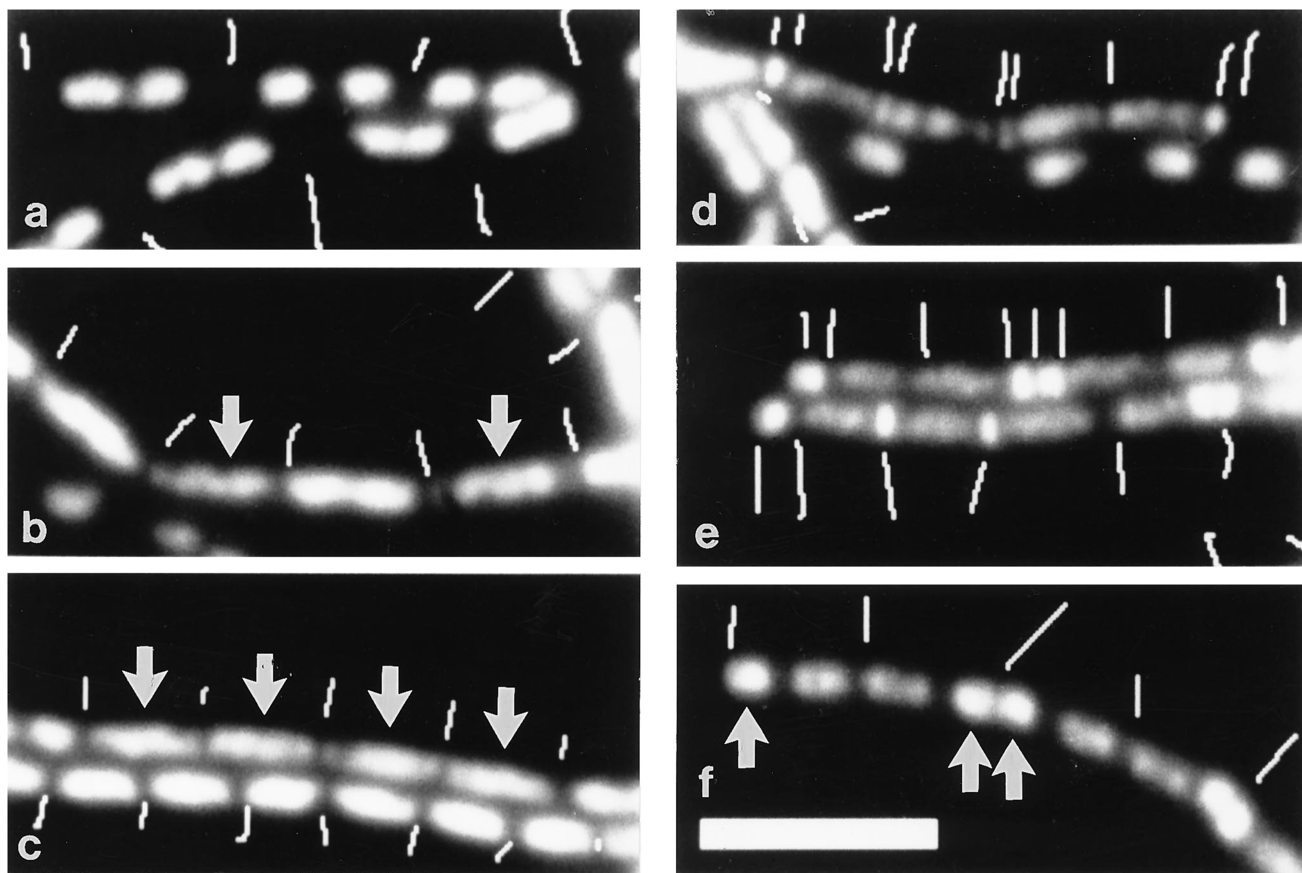

FIG. 6. Classification of cells after resuspension, according to the appearance of their nucleoids stained with DAPI. Strain SG38 was grown in CH medium, resuspended in SM, and analyzed. Pictures of samples taken 0 (a), 45 (b), 60 (c and d), and 90 (e and f) min after resuspension were obtained by the procedure described in the legend to Fig. 2. At 0 min (a), only mononucleate and binucleate cells were observed. At 45 (b) and 60 (c) min, besides mononucleate and binucleate cells, cells that had reached the preseptation stage, with less condensed DNA, were present (arrows). At 60 min (d), the majority of the sporulating organisms did not show condensed prespore DNA because its segregation occurs after septation (see text). Closely spaced septa demarcate the positions of prespore compartments. At 90 min (e and f) as well as at 75 min (Fig. 2), sporulating organisms with condensed prespore DNA were observed. At 90 min (f), in some cells prespore engulfment had occurred (arrows). In panels b and d, two and four standard round or ovoid nucleoids are visible, respectively. All pictures are at the same magnification. Scale bar, 5 μm.

showed similar stages of morphological development in about 85% of the cell pairs, at both time points.

DISCUSSION

The sensitive stage of the cell cycle. The idea that there is a sensitive stage in the DNA replication cycle, with respect to the initiation of sporulation, has been invoked in several publications (7, 11, 29). Dunn et al. (11) showed that sporulating cells begin to escape from the effects of hydroxyphenyl azo-uracil (HPUra), which immediately and specifically inhibits DNA replication, at 35 ± 4 min after resuspension (confirmed more recently in references 17 and 18). Escape occurs when cells that are the most advanced in the sporulation process complete the final round of DNA replication needed to produce the prespore and mother cell chromosomes. Since the round of replication takes about 55 min and since this time is not affected by resuspension in SM (Fig. 5), the first cells to escape HPUra inhibition must have initiated this final round of DNA replication 20 min before resuspension. According to our model for the average cell cycle in CH medium, the sensitive stage must therefore occur about 15 min after division (Fig. 4).

What is the nature of the sensitive stage? Mandelstam and Higgs (29) suggested the existence of a gene, located about

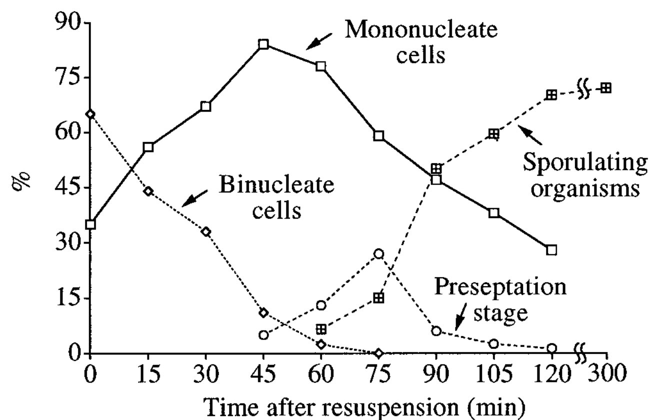

FIG. 7. Variation in the proportions of cell classes after induction of sporulation. Strain SG38 was grown in CH medium, resuspended in SM, and analyzed. Cells were classified according to the appearance of their nuclei (see text and Fig. 2 and 6). Between 89 and 208 cells per sample were analyzed.

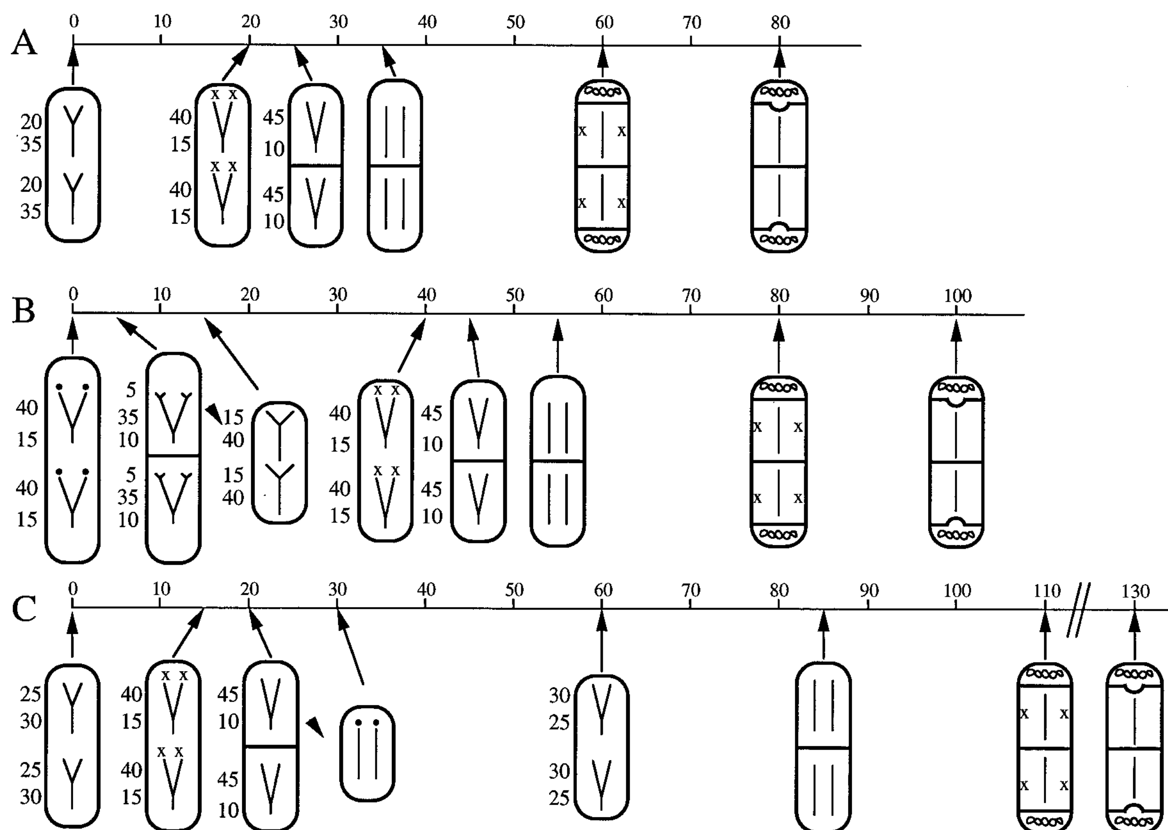

FIG. 8. Model for cell cycle events following the induction of sporulation. Shown are predicted fates of cells at different points in the cell cycle in CH medium, following resuspension in SM (Fig. 4). (A) Cell that is just about to reach the sensitive stage in the cell cycle. (B) Cell that has just initiated a new round of DNA replication. (C) Cell that has just passed the sensitive stage. Assumptions made in relation to the length of the period between completion of DNA replication and the next septation and the DNA replication time in SM are discussed in the text. Crosses stand for inhibition of reinitiation of DNA replication (in relation to the time that this would have occurred had the cells remained in CH medium) or of central division. Prespore DNA is shown as a condensed structure. The final morphological event shown, characterized by the bulging of the prespore septum, represents the onset of prespore engulfment. Other symbols are as described for Fig. 4.

one-third of the chromosome from the origin of replication, which must be replicated under the starvation conditions for sporulation to be initiated. Such coupling of replication with transcription has been described for bacteriophage T4 and transposons Tn*5* and Tn*10* (see reference 23 for references). Mutations in such a gene should produce a nonsporulating phenotype. Unfortunately, none of the *spo0* genes identified so far map in the appropriate region of the chromosome (1).

Our results suggest a new interpretation of the sensitive stage. The strong correlation found between DNA content and cell size (Fig. 3) and the relatively uniform size of sporulating organisms (see above) suggest that the sensitive stage could represent a specific cell size rather than a specific state of DNA replication. We suggest that beyond the sensitive stage, the cell could be too large to avoid reaching the initiation mass for DNA replication (10), even at the two- to threefold slower growth rate in SM, and thus becomes committed to another round of DNA replication (see below). The possibility that the initiation of DNA replication is blocked as an indirect consequence of the nutritional downshift is attractive because this does not require any new sporulation-specific mechanism of inhibition. However, we cannot rule out the possibility that the changes in transcription associated with the onset of sporulation (22) effect a direct block in DNA replication.

Cell cycle changes during the initiation of sporulation. The behavior of a cell that is resuspended in SM just before the sensitive stage is modelled in Fig. 8A, incorporating information from previous electron microscopic studies of the timing of septation and a subsequent morphological event, prespore engulfment (33). The cell completes its round of DNA replication after 35 min (thus escaping from HPUra inhibition). Twenty-five minutes later, it makes an asymmetric septum, and after 80 min, engulfment of the prespore begins. How do cells at other points in the cell cycle behave? Cells with replication forks between the origin of replication (cells which have just initiated a round of replication) and the sensitive point (indicated by the shaded semicircle in Fig. 4) would complete their ongoing rounds of DNA replication, escape from HPUra inhibition, and presumably follow on behind the cell shown in Fig. 8A. The likely behavior of a cell at the beginning of this stage of the cycle, which would have just initiated a new round of DNA replication at the time of resuspension, is shown in Fig. 8B. All of the events indicated in Fig. 8B should occur about 20 min after those for the cell depicted in panel A. The behavior of the remaining cells, which apparently need to initiate a new round of DNA replication in SM, is more difficult to predict, because the time needed to reach the putative initiation mass is unknown: after transfer to SM, the growth rate gradually tails off and the mass of the culture eventually doubles only after about 2 h. In Fig. 8C, the behavior of an extreme example of this class of cell is modelled. This cell belongs to a class which had just passed the sensitive stage of the cell cycle at the time of resuspension (Fig. 4). If this cell had remained in CH medium, it would have initiated a new

round of DNA replication after 15 min in SM. After 30 min, it completes its ongoing round of DNA replication. It is not inconceivable that at about the same time, it reaches the initiation mass for a new round of replication. Commitment to a new round of DNA replication would preclude the cell from forming a spore septum until about 110 min after resuspension, as shown. We thus suggest that it is the commitment to a new round of DNA replication that precludes cells that have passed the sensitive point in the DNA replication cycle from sporulating.

Our direct observations of a population of cells induced to sporulate by resuspension (Fig. 7) accord well with various predictions arising from the data shown in Fig. 8. First, according to Fig. 8, the preseptation state should begin to appear after about 35 min and the majority of cells should have left this state by 80 min, with a tailing off extending until about 110 min (Fig. 8C). Second, the preseptational cells should be replaced by cells with asymmetric septa (stage II of sporulation) beginning about 60 min after resuspension. It is evident that the kinetics of the preseptation state adhere well to both of these predictions (Fig. 7). In addition, since most cells contained four origins of replication at the moment of resuspension (Fig. 4), sister cell pairs should tend to be synchronized with respect to their state of development. As mentioned above, virtually all cell pairs observed (84%) showed similar states of development. The synchronization of sporulation in sister cells has been reported previously but in relation to relatively late events, from septation onwards (9). In this study, we found strong synchronization even when examining early, relatively transient events.

Asymmetric septation. The last central septation, which should have followed from completion of the last round of DNA replication initiated before resuspension, needs to be blocked soon after induction of sporulation (12) (Fig. 8). Some *spoO* mutations may be located in the genes required for this inhibition since, after being induced to sporulate, such mutants produce cells about half the size of sporulating organisms (12, 15, 35). Interestingly, the time between the completion of DNA replication and septation in CH medium (30 min; Fig. 4) is close to the analogous period observed after initiation of sporulation (25 min; Fig. 8). Initiation of sporulation therefore does not change the timing of septation, only the location of the septum in the cell. This observation strongly supports the view that spore septum formation is a modified version of proliferative cell division (20, 21). The important challenge that remains is to understand how the position of the septum is diverted from its normal midcell position to the pole.

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