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Chromosome Replication in Sporulating Cells of *Bacillus subtilis*

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A method of specifically labeling the chromosomal terminus of *Bacillus subtilis* is described. When sporulating cultures were pulse-labeled with [^{3}H]thymidine and then treated with 6-(*p*-hydroxyphenylazo)uracil, a drug which inhibits deoxyribonucleic acid (DNA) synthesis rapidly and completely, the only labeled spores formed were those that had completed replication during the pulse period. DNA-mediated transformation was used to show that the DNA of spores formed in the presence of 6-(*p*-hydroxyphenylazo)uracil had the same ratio of origin to terminus markers as DNA from untreated spores. Furthermore, spores formed in the presence of 6-(*p*-hydroxyphenylazo)uracil had the same DNA content as untreated spores. These two observations indicated that spores formed in the presence of 6-(hydroxyphenylazo)uracil contained completed chromosomes. The rate of termination of chromosomes destined to be packaged into spores was determined by this method, using the Sterlini-Mandelstam replacement system and a single medium exhaustion system for inducing sporulation. With both systems the rate of termination reached a broad peak 2 h after the start of sporogenesis. This was measured from the time of resuspension by using the replacement system and from the point where exponential growth ceased in the exhaustion system. The amount of spore DNA synthesized in the Sterlini-Mandelstam sporulation-inducing medium was very close to one-half the amount of the DNA present in mature spores. This suggests that chromosomes destined to be packaged into spores were replicated from close to the origin and possibly initiated in the sporulation-inducing medium. A method was devised for estimating the time taken to complete replication of the chromosomes destined to be packaged into spores. This was probably no more than 50 min. Whereas starvation must have occurred almost simultaneously in most cells in the population, the chromosome replication that was essential for sporogenesis was distributed over a wide time span. Thus, in some cells, replication started within 10 min of the nutritional step-down, but the peak rate was not reached for 1 h; thereafter replication continued at a substantial rate.

It is widely believed that the chromosomes which are going to be packaged into spores are replicated during the early phase of sporogenesis in *Bacillus subtilis* 168 and that complete replication of a potential spore chromosome is a necessary condition for sporogenesis (6, 20). The first evidence that spores of *B. subtilis* may contain completely replicated chromosomes was obtained by Oishi et al. (23). These authors inferred from the order of replication of genes in outgrowing spores and vegetative cells that spores contain completely replicated chromosomes. Later, it was suggested that spores of Thy strains may contain incomplete chromosomes, as they appeared to have anomalous ratios of origin to terminus markers, when DNA-mediated transformation was used (11, 13). The number of genomes per spore has also been studied by using autoradiography of outgrowing spores, but the conclusions have been disputed by those who believe that each spore has one (5) or two genomes (36) or that spore populations contain a mixture of haploid and diploid cells (1, 26). However, the most recent studies indicate strongly that there is only one genome per spore (5). The apparently anomalous gene frequencies found in spores of Thy$^-$ strains are probably not correct, as the efficiency of integration of one of the markers (i.e., *metB*) varies depending on its source. The Thy$^-$ mutations originated from *B. subtilis* W23 and were transformed into strain 168, possibly with other genes from the terminus of W23 (4).

The earliest phase of spore formation is dependent on DNA synthesis. Thus, spore formation does not occur in *B. subtilis* 168 Thy$^-$ when bacteria are suspended in a sporulation-inducing medium (SM) that lacks thymine. With increas-

ing time in the SM in the presence of thymine, a progressively larger fraction of the population can proceed to form spores if the thymine is removed (20). Some workers have used the drug 6-(*p*-hydroxyphenylazo)uracil (HPU), which is a specific inhibitor of DNA polymerase III (3, 17, 18), to determine the point at which sporogenesis becomes independent of DNA synthesis (10, 17, 29, 37).

It has been suggested that when cultures of *B. subtilis* are challenged by a sporulation-inducing stimulus, the only cells which can respond are those from a restricted part of the cell cycle (7). Experiments with chromosome initiation mutants suggested that this may correspond to a particular region of the chromosome which is about 30% from the origin (19). However, Dunn et al. (10) have provided evidence which suggests that chromosome initiation is likely to occur in an SM. Thus, vegetative cells of *B. subtilis* with completely replicated chromosomes (obtained in the presence of chloramphenicol) form spores when transferred to the Sterlini-Mandelstam SM. Studies with HPU indicate that DNA synthesis is required in the SM in order to obtain spores, so it is likely that chromosome initiation occurred in that medium.

The relationship between sporulation and cell cycle has also been studied by using microcycle sporogenesis (i.e., production of spores from spores without intervening cell division [21, 22]). Microcycle sporogenesis can be obtained in *Bacillus megaterium* by transferring germinating spores into an SM. The capacity to form spores on transfer to an SM reaches a peak during outgrowth of the germinating spores at approximately the start of chromosome replication, when the rate of peptidoglycan and phospholipid synthesis starts to increase exponentially. The relationship between commitment to sporulation and the chromosome cycle has also been investigated by using germinating spores of *B. subtilis* (15). Germinating spores have no significant capacity to sporulate on transfer to an SM until the chromosomes are well into their second round of replication. Keynan et al. (15) suggest that in order to start sporogenesis, a cell must contain two completely segregated chromosomes (i.e., two chromosomal termini). *Bacillus cereus* and *B. megaterium*, which are able to undergo microcycle sporogenesis without cell division, have spores containing two chromosomes, so the requirement of two nuclei per cell is met before the first division occurs during outgrowth (15).

Attempts to study the replication of DNA destined to be packaged into spores have been rare, although the time course of DNA replication in sporulating cultures has been described many times. Furthermore, clear distinctions have not been made between vegetative DNA synthesis and DNA synthesis which occurs after the sporulation process has started. Fitz-James and Young (12) concluded that most of the DNA found in spores was synthesized during vegetative growth. Canfield and Szulmajster (6) estimated the amount of DNA synthesized during sporogenesis by using the rate of $^{32}P_i$-induced "suicide" of a heat-stable cell population. This suggested that the DNA packaged into spores in an exhaustion system of sporogenesis was synthesized no more than 90 min after the first deviation from exponential growth.

The purpose of this paper is to describe a more direct method of studying the replication of DNA destined to be packed into spores.

MATERIALS AND METHODS

Bacteria. The following strains were used: 168 *trpC2* and 168 *trpC2 thyA thyB*. An *ilvA1* derivative of strain SL35 (24) was used for transformation experiments.

Induction of sporulation. All cultures were grown with aeration at 35°C.

Method A: Sterlini-Mandelstam procedure. In the Sterlini-Mandelstam procedure (32), bacteria were grown to about 600 μg (dry weight) per ml (optical density at 540 nm, 3.0) in growth medium (GM) and then suspended at 200 μg/ml in SM. Incubation was continued for 20 h.

Method B: single medium exhaustion system. A mixture of GM and SM (ratio, 1:5) containing 0.01 M magnesium sulfate was used. GM and SM differ in their concentrations of $MgSO_4$ (0.8 and 40 mM, respectively) and $CaCl_2$ (0.2 and 1 mM, respectively) but otherwise have essentially identical salt compositions. GM contains casein hydrolysate, alanine, asparagine, and glutamate, whereas SM contains only glutamate. Therefore, the single medium system differs significantly from SM only in its content of alanine (0.2 g/liter), asparagine (0.23 g/liter), and casein hydrolysate (1.67 g/liter). This medium was inoculated with a culture grown on GM. The culture could be maintained in exponential growth indefinitely (generation time, 50 to 60 min) without significant sporulation. The first deviation from exponential growth occurred at an optical density at 540 nm of 0.75, which was regarded as the start of sporogenesis (i.e., T_0). Cultures were incubated for 20 h after the end of exponential growth.

Measurement of growth. Samples (0.5 ml) were transferred to an equal volume of 10% Formalin, and the optical density at 540 nm was determined with a Unicam SP500 spectrophotometer. This gave a linear response up to an optical density of 1.0 (equivalent to 200 μg, dry weight). Samples were diluted appropriately to be in the linear range.

Spore counts. Viable counts of spores were made by plating onto nutrient agar after heat treatment at 80°C for 10 min. Purified spores were also counted

with a Coulter electronic particle counter. Counts performed on the same sample by the two methods usually were in good agreement. Furthermore, when the time course of increase in HPU-resistant spores was determined by using either viable counts of spores or particle counts of purified spores, the curves obtained could be superimposed within an error of no more than 20%. If HPU is added to cultures induced to sporulate by method A at 4 h after resuspension, the number of heat-resistant spores per milliliter formed after 20 h is approximately 90% of the number of spores found in an untreated culture, as noted by Dunn et al. (10).

Isolation of spores. After completion of the sporulation period, the bacteria were sedimented, suspended in 0.02 M Tris-hydrochloride (pH 8.0)–0.01 M EDTA, and treated with lysozyme (200 μg/ml) at 35°C for 30 min. After 1% sodium dodecyl sulfate was added, the lysate was sheared vigorously with a Vortex mixer, and the spores were sedimented. These spores were layered onto 2 ml of 45% Urografin (in 0.1% sodium dodecyl sulfate) and spun in a 3× 5-ml swing-out rotor at 20,000 rpm for 20 min. Heat-resistant spores were recovered quantitatively in the sediment. The spores were washed in distilled water, and their purity was checked by phase microscopy. The procedure was repeated if the preparation was contaminated with bacilli. A second criterion of purity was provided by the size distribution given by a Coulter Channelyser. Samples of spores to be counted were diluted in 0.1% sodium dodecyl sulfate to prevent clumping. When pure spore preparations were used, this method gave a highly reproducible major symmetrical peak of 0.38 nm^3 with a coefficient of variation of 5.3% and a very small peak of twice the volume, which was assumed to represent doublets. Any significant bacillary contamination would have altered this pattern.

Radioactive methods. (i) Rate of DNA and RNA syntheses. Samples of cultures (0.5 ml) were transferred to tubes (internal diameter, 2.5 cm) containing 1 μCi of [*methyl*-^{3}H]thymidine (specific activity, 52 Ci/mmol) or [6-^{3}H]uracil (112 Ci/mol), which were incubated at 35°C with shaking. The pulse was terminated after 5 min with 2 ml of ice-cold 5% trichloroacetic acid containing nonradioactive thymidine or uracil (100 μg/ml).

(ii) Steady-state labeling of DNA. *B. subtilis thy* was grown overnight (at least 10 generations) in the presence of 20 μg of [^{14}C]thymine (3.15 Ci/mol) per ml. It has been shown previously that the sole source of thymine in the DNA of this strain is from the medium (27).

(iii) Pulse-labeling of chromosomal termini. Volumes (10 ml) of sporulating cultures were transferred to flasks containing [*methyl*-^{3}H]thymidine (usually to give 1 μCi/ml). After 10 min, the pulse was terminated by adding 50 μg of HPU per ml. After incubation for 20 h, the spores formed were purified, and the concentration of spores and the radioactivity in the spores per milliliter of culture were determined.

Extraction of DNA from spores. DNA was extracted from pure spore preparations by the method of Sargent (28).

Transformation. Competent bacteria were obtained by the method of Wilson and Bott (35). Samples of competent cells were added to test tubes containing approximately 0.1 μg of DNA and 1 ml of competence medium and were incubated with shaking for 1 h. On completion, DNase (10 μg/ml) was added, and each culture was diluted with minimal medium (30) supplemented with glucose (5 mg/ml), ferrous sulfate (1 μg/ml), and manganese sulfate (0.1 μg/ml) and plated onto selective media. Ratios of markers were based on at least 1,000 transformants of each kind. The number of *metB5* transformants was typically between 2×10^5 and 5×10^5 transformants per ml of competent cells.

RESULTS

Effect of HPU on sporulation. When HPU is added to sporulating cultures or vegetative cultures at 50 μg/ml, DNA synthesis is instantly reduced to about 1% of the control rate (3, 10, 17). This drug is thought to prevent sporulation in cells engaged in chromosome replication but to permit sporulation in cells in which replication has been completed (10, 17, 29, 37). As it has been suggested that incomplete chromosomes may be packaged into spores (11), I sought direct evidence to show that spores formed in the presence of HPU contain completed chromosomes by using the method of marker frequencies (Table 1). Sporulation of *B. subtilis* Trp$^-$ Thy$^-$ was induced by transfer of cells to SM (method A). HPU was added to part of the culture in SM at 120 min, as less than one-half of the maximum possible spore population had completed chromosome replication at this time (Fig. 1A). The genes used for transformation studies were *purA16* (origin), *argC4* (mid-chromosome), *metB5* (terminus), and *ilvA1* (terminus). Table 1 shows frequencies of these genes in spores relative to the frequency of *met-B* transformants obtained under the two conditions in two independent experiments. There was no significant difference between treated and untreated spores, which suggests that like untreated spores (4), HPU-treated spores contain completed chromosomes.

Labeling of the terminus in the Sterlini-

TABLE 1. *Marker frequencies in spores prepared in the presence of HPU*[a]

Expt	Treatment	Frequency of transformants relative to *metB*		
		purA16/ metB5	*argC4/ metB5*	*ilvA1/ metB5*
1	Untreated	1.16	1.11	0.51
	HPU present	1.06	1.16	0.52
2	Untreated	0.97	1.03	0.31
	HPU present	1.0	1.25	0.30

[a] Sporulation of *B. subtilis* 168 Trp$^-$ Thy$^-$ was induced by transfer of cells to SM (method A). HPU was added to part of the culture in SM at 120 min. Spores were purified after 20 h.

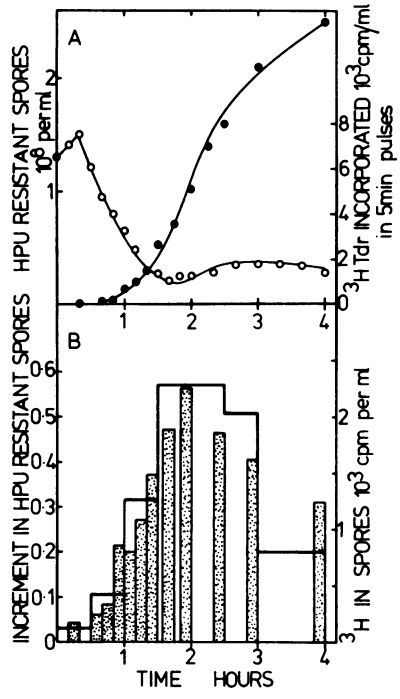

FIG. 1. *Labeling of the chromosomal terminus of B. subtilis 168 Trp⁻ Thy⁻ during sporogenesis by method A. Bacteria grown in GM were suspended in SM. (A) Symbols: ●, number of cells per milliliter committed to form spores after 20 h in the presence of HPU; ○, rate of DNA synthesis per milliliter of culture measured as amount of [^{3}H]thymidine (^{3}H Tdr) incorporated into trichloroacetic acid-insoluble material in 5 min. (B) The stippled columns show the rate of chromosome termination measured as amount of radioactivity incorporated into potential spore-forming cells in 10 min per milliliter of culture. Pulses were termined with HPU at the times shown. The open areas show the rate of chromosome termination measured from the HPU escape curve (Fig. 1A) as the increment in number of cells committed to form spores in the absence of DNA synthesis per milliliter during each 30-min time interval.*

Mandelstam replacement system. If HPU acts on DNA synthesis instantaneously and incomplete chromosomes are not incorporated into spores, then it follows that when cultures in an SM are pulse-labeled and then treated with HPU, the only spores which should be labeled are those in which replication was completed during the pulse period. Furthermore, if the pulse is sufficiently short, the DNA of these spores should be labeled only in the terminal region, providing that replication proceeds in the normal sequential semiconservative fashion. This seems likely, as DNA synthesis in sporulating cells is as sensitive to HPU as is DNA synthesis in vegetative cells. The kinetics of termination of chromosomes destined to be packaged into spores were examined by this procedure and compared with the rate of DNA synthesis in a sporulating culture. Sporulation of strain 168 Trp⁻ Thy⁻ was induced by method A. The rate of termination of chromosomes destined to be packaged into spores was determined as described above (Fig. 1). As shown by others (10, 17, 29, 37), sporulation is initially completely sensitive to HPU, and thereafter the number of bacteria which can form spores in the absence of DNA synthesis increases gradually (Fig. 1A). Figure 1B shows the amount of radioactivity in spores per milliliter of culture. This represents the rate at which potential spore chromosomes were completed during spore induction. As Fig. 1B shows, the rate of termination of chromosomes destined to be packaged into spores was low for 50 min, after which there was an increase to a peak at about 120 min; thereafter the rate fell slowly for several hours, but it was considerable even after 4 h. An almost identical pattern of labeling was obtained when strain 168 Trp⁻ was used. The rate of termination contrasted sharply with the rate of DNA synthesis in the whole culture, which included sporulating and nonsporulating cells (Fig. 1A). The rate of DNA synthesis fell continuously and reached about 15% of the initial level after 1 h; it then remained almost constant for several hours.

The increment in number of cells per milliliter committed to form spores during each 30-min time interval was calculated from the time course of the increase in HPU-resistant spores (Fig. 1A) (i.e., the difference between successive 30-min points [Fig. 1B]). Although the curve shown in Fig. 1A is fairly smooth, the calculated rate of commitment to sporulation from this data is inherently less accurate than a rate measurement. However, the two measurements are in reasonable agreement.

Labeling of the terminus in an exhaustion system. Certain strains of *B. subtilis* 168 which sporulated poorly when the Sterlini-Mandelstam procedure was used sporulated massively when grown in the single medium exhaustion system (method B). All of the strains of *B. subtilis* 168 examined grew exponentially at low cell densities with very few if any spores and had a generation time of 50 to 60. After reaching the end of exponential growth, the bacteria started to make spores. The start of sporogenesis (T_0) was taken as the end of exponential growth (Fig. 2). At this time the rate of DNA and RNA syntheses reached a peak and thereafter decreased (Fig. 3A). No attempt was made to determine the specific activities of the triphosphate precursors of DNA and RNA. Similar changes in the rate of uracil incorporation have

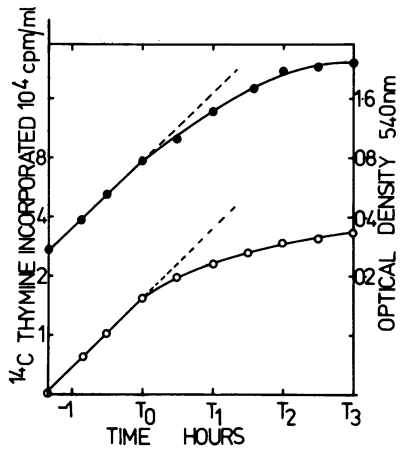

FIG. 2. *Time course of mass and DNA accumulation in B. subtilis 168 Trp$^-$ Thy$^-$ during sporogenesis when method B was used. Symbols: ●, time course of optical density increase at 540 nm; ○, time course of [^{14}C]thymine incorporation into trichloroacetic acid-insoluble material. The point at which the mass growth curve starts to deviate from exponential growth is defined as T_0.*

been reported by others (2, 14, 31, 33). The rate of DNA synthesis fell rather abruptly after T_0 and was a slightly better indicator of the start of sporulation. The most likely explanation for this behavior is that the rate of chromosome initiation decreased almost as soon as the nutritional step-down occurred, whereas the rate of replication continued unchanged.

The rate of termination of chromosomes destined to be packaged into spores was determined as described above (Fig. 3). The number of cells committed to making spores in the presence of HPU started to increase about 1 h after the end of exponential growth and increased most sharply between 90 and 120 min. Figure 3B shows the number of cells per milliliter committed to form spores during each 30-min time interval, together with the rate of termination. As with sporulation induced by method A, these two values were in reasonably good agreement. Completion of chromosomes destined to be packaged into spores started at 1 h after T_0 and reached a peak between 1.5 and 3 h after T_0, after which the rate of termination started to decrease. Almost identical results were obtained when strain 168 Tryp$^-$ was used.

Origin of spore DNA in the Sterlini-Mandelstam system. When spores were produced by the Sterlini-Mandelstam procedure, approximately one-half of the spore DNA was synthesized in the induction medium (SM). This was demonstrated (Table 2) by transferring *B. subtilis* 168 Trp$^-$ Thy$^-$ steady-state labeled with [^{14}C]thymine in GM to SM containing either [^{14}C]thymine of the same specific radioactivity (see above) or nonradioactive thymine (10 μg/ml). The reduction in specific radioactivity of spores obtained in nonradioactive thymine gave the proportion of the chromosomes made during vegetative growth. Both cultures were divided into two parts, one of which was treated with HPU at 90 min; the other was not treated. Table 2 shows the specific radioactivities obtained in the spores formed after 20 h. There was no significant difference between the amount of DNA in untreated spores and the amount of DNA in spores prepared in the presence of HPU. An average value of 5.4 ± 0.44 μg of DNA per 10^9 spores was obtained in five experiments. This is in good agreement with previously published

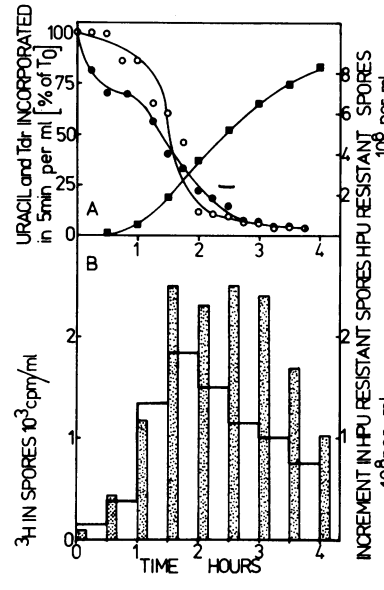

FIG. 3. *Labeling of the terminus of B. subtilis 168 Trp$^-$ Thy$^-$ during sporogenesis by method B. Bacteria were grown in a mixture of GM and SM (1:5). Zero time is equal to T_0 in Fig. 2. (A) Symbols: ■, number of cells per milliliter committed to form spores after 20 h in the presence of HPU; ○ and ●, rates of RNA and DNA syntheses, respectively, per milliliter of culture, measured as amounts of [^{3}H]uracil and [^{3}H]thymidine (Tdr) incorporated into trichloroacetic acid-insoluble material in 5 min (both shown as percentage of T_0). (B) The stippled columns show the rate of chromosome termination measured as amount of radioactivity incorporated into potential spore-forming cells in 10 min per milliliter of culture. Pulses were terminated with HPU at the times shown. The open areas show the rate of chromosome termination measured from the HPU escape curve (Fig. 1A) as the increment in number of cells committed to form spores in the absence of DNA synthesis per milliliter during each 30-min time interval.*

TABLE 2. *Origin of spore DNA*[a]

Label present in:		HPU added at 90 min	cpm/10^8 spores	% Of control[b]
GM	SM			
+	+	−	2,094	100
+	−	−	1,097	52
+	+	+	2,080	99
+	−	+	1,161	55

[a] *B. subtilis* Trp⁻ Thy⁻ was grown in GM in the presence of 2 μg of [^{14}C]thymine (specific activity, 3.15 Ci/mol) per ml. Sporulation was induced by suspension in SM containing either [^{14}C]thymine or nonradioactive thymine.

[b] The control was the culture which was labeled in both GM and SM and to which HPU was not added at 90 min (2,094 cpm/10^8 spores).

values (1, 9, 12). The specific radioactivity of spores completed in medium containing nonradioactive thymine (with or without HPU) was just over 50% of that of steady-state labeled spores (Table 2).

Chromosome replication time in sporulating cells. It cannot be assumed that the final round of replication before spore formation occurs at the same rate as in vegetative cells (10). The time required to complete replication under sporulating conditions was determined as follows. By treating the sporulating culture with HPU early in the process of spore formation such that only a small proportion of the maximum possible spore yield was obtained (i.e., at 90 min [Fig. 1A]), the investigation was restricted to a population of chromosomes that terminated over a narrow range of time. In the experiment reported below and in almost all others performed, more than 75% of the spores that were able to sporulate when HPU was added at 90 min had terminated chromosome replication in the preceding 20 min. Thus, if such a culture was labeled for 30 min before HPU was added, the spores obtained were labeled to varying extents, ranging from short lengths in those that terminated early in the pulse to those which were labeled throughout the period. A further increase in the labeling period increased the specific activity of the spores until the DNA which could be labeled in the SM was labeled completely. This period was equal to the replication period in the SM plus the period over which termination was distributed (in this case about 20 min). Samples of a sporulating culture (10 ml) (Fig. 4) were transferred to flasks containing [^{3}H]thymidine (5 μCi) at frequent intervals between 0 and 90 min. At 90 min HPU was added to all of the flasks, and incubation was continued for 20 h. The spores were then isolated and the specific radioactivity per 10^8 spores was determined. This radioactivity is plotted in Fig. 4 against the time at which the pulse was begun. During the first 40 min there was little change in the amount of label, thus indicating that spore DNA was not synthesized during this time. After this point the radioactivity fell sharply, on a line which extrapolated to about 85 min. The latter part of the curve leveled off to pass through zero at about 90 min. This observation suggests that DNA which was to be incorporated into spores was synthesized in no more than 50 min. As noted above, there was a distribution of termination times in the period before HPU was added, so that the inflection point at 40 to 50 min overestimated the replication period. The maximum possible replication period was therefore 50 min, although 40 min seems to be a more likely value.

DISCUSSION

I describe above a method for determining the rate of termination of chromosomes destined to be packaged into spores and for specifically labeling the terminus. The specificity of labeling was confirmed (unpublished data) by showing that about 25% of the radioactivity incorporated into the terminus in a 10-min pulse hybridizes with the DNAs of phages (SPβ and ϕ3T) whose prophages are integrated at the terminus. Furthermore, this DNA has a limited number of labeled restriction fragments (unpublished data).

When the resuspension method is used, the start of sporogenesis is unambiguous, but the timing of DNA replication may be perturbed by the abrupt change in medium. With the single medium exhaustion system this is not a problem, but T_0 cannot be determined so precisely. How-

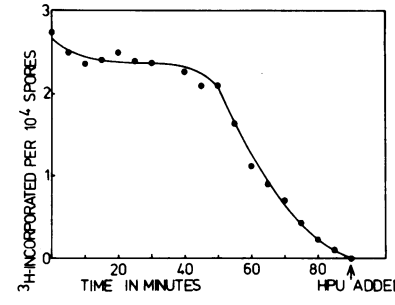

FIG. 4. *Time taken to replicate chromosomes in sporulating cells. Sporulation was induced in B. subtilis 168 Trp⁻ Thy⁻ by method A. Samples (10 ml) were transferred to flasks containing 5 μCi of [^{3}H]thymidine at the times shown, and HPU (50 μg/ml) was added to all flasks at 90 min. Each point represents the radioactivity in purified spores harvested after 20 h.*

ever, if the point at which the rate of synthesis of RNA and DNA starts to fall is taken as T_0, the timing of replication of chromosomes destined to be packaged into spores is strikingly similar. In both systems, the median time of termination after T_0 is 127 min, with a standard deviation of 20 min, and there is a considerable rate of termination 4 h after T_0.

Almost exactly one-half of the DNA in the spores produced by the Sterlini-Mandelstam procedure was synthesized in SM. This can be interpreted in the following two ways: (i) one complete strand is labeled, or (ii) a short distal part of the template strand and a large part of the daughter strand are labeled. If the latter is true, the semiconservative replication process must be interrupted during the change of medium and not resumed for at least 40 min on average. At present, this seems improbable, as in general an interruption of bacterial DNA synthesis is lethal. It seems plausible therefore that chromosomes are actually initiated in the SM. A more incisive experiment would be required to prove that the DNA synthesized in the SM represents one complete strand. Whether the spore chromosome was completed early or late during growth in SM, one-half of the DNA present was synthesized in SM. This suggests that regardless of the time at which replication of the spore chromosome is completed, the template strand is always of vegetative origin.

The chromosomal replication time of 45 to 50 min obtained by Dunn et al. (10), who used the marker frequency method for *B. subtilis* grown in GM, has been confirmed, and a similar value was obtained for the period of DNA synthesis required for chromosomes destined to be packaged into spores. The rate of spore chromosome initiation would be expected to follow a time course similar to that for termination but 40 to 50 min earlier. Calculated in this way, in both systems of sporogenesis, the first initiations must occur within 10 min of the nutritional step-down, but the peak rate of initiation occurs at 70 min and continues at a substantial rate for at least 3 h.

The data of Dunn et al. (10) for the onset of resistance to HPU are also consistent with the idea that some chromosome initiation occurs in SM. Moreover, these authors obtained spores after they resuspended in SM a culture which had been allowed to complete replication in the presence of chloramphenicol. Studies of HPU inhibition of sporulation in this system suggested that sporulation became independent of DNA synthesis between 2 and 4 h after resuspension. Studies of microcycle sporogenesis in *B. megaterium* (21, 22) suggested that induction of sporulation cannot occur until germinating spores reach the point at which chromosome initiation normally occurs. It is not clear whether initiation occurs in the germination medium or the SM.

The nature of the trigger that induces chromosome initiation in sporulating cultures is not known. Chromosome initiation in *Escherichia coli* may be related to the attainment of a critical cell mass (25), so that the rate of initiation would be sharply reduced under nutritional step-down conditions. The reduced rate of DNA synthesis after T_0 presumably results from a reduction in the rate of initiation. Dunn et al. (10) noted a reduction in the ratio of origin to terminus markers after T_0, which indicated that chromosomes were being completed with comparatively little initiation. It is difficult to judge from the data available whether initiation (if it is indeed initiation from the normal vegetative origin) of the potential spore chromosome is activated by the attainment of a critical cell mass. The rate of mass growth is greatly reduced by the time of initiation, but initiation is delayed for two generation times after T_0. de Lencastre and Piggot (8) have observed spore formation which is dependent on DNA synthesis under sporulation-inducing conditions where there is extremely little mass growth (P. J. Piggot, personal communication).

The nutritional step-down which presumably inaugurates sporogenesis must occur almost synchronously in all cells in a population, whereas, in sharp contrast, initiation of spore chromosomes is distributed over a wide time scale. Possibly these initiations are scheduled by a clocklike process that is a normal feature of vegetative growth and which is in some way related to cell age. This could be the source of the distribution of times at which spore septum formation (stage II) is reached (34). It seems plausible that when initiations occur after the onset of starvation, the chromosomes are "modified" by a special mechanism so that they participate in sporogenesis. Young and Jeffs (37) have shown that if the rate of chromosome replication is reduced so that termination is delayed during the early phase of sporogenesis, the time of appearance of markers such as alkaline phosphatase and protease is not affected. The timing of these events is therefore not dependent on chromosome replication.

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