Sporulation in *Bacillus subtilis*

EFFECT OF MEDIUM ON THE FORM OF CHROMOSOME REPLICATION AND ON INITIATION TO SPORULATION IN *BACILLUS SUBTILIS*

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Thymine-requiring mutants of *Bacillus subtilis* and mutants that are temperature-sensitive for initiation of chromosome replication have been used to study the relationship between sporulation and chromosome formation. The DNA synthesis that normally occurs when cells are transferred to sporulation medium is essential for spore induction. This is shown by the fact that thymine-starved cells are unable to form spores and are unable to perform even the earlier steps of sporulation, such as septum formation or synthesis of alkaline phosphatase. The nature of the medium in which the cells are growing while the DNA is being completed is also important because it determines both the shape and the position of the daughter chromosomes. If the cells are in a rich medium, the newly synthesized chromosomes are discrete and compact bodies: the cells are primed for growth, and sporulation cannot be induced by transferring them at this stage to a spore-inducing medium. If DNA synthesis was completed with the cells in a poor medium the daughter chromosomes, by the time DNA synthesis has ceased, are spread in a single filamentous band and the cells are morphologically already in stage I of sporulation.

Dawes & Mandelstam (1970) reported that when a chemostat culture of *Bacillus subtilis* was subjected to repeated half-hour periods of acute starvation, each such period induced sporulation in only a fraction of the cells that would have been induced by more prolonged treatment. The result was a stepwise increase in spore incidence up to a plateau value. From these experiments it was tentatively concluded that there was a sensitive stage in the cell division cycle during which induction of sporulation could occur, and that each period of starvation caught a fraction of the cells in this state. Further, the genetic work of Oishi, Yoshikawa & Sueoka (1964) implied that spores of *B. subtilis* contain chromosomes that are complete (i.e. that have no replication forks) so it appeared that partly replicated chromosomes cannot be enclosed in the developing spores.

Because of these findings we assumed as a working hypothesis that sporulation could be induced in a cell only when it had finished replicating its chromosome. This assumption is also consistent with the radioautographic studies of Ryter & Aubert (1969) on sporulating cells. The availability of thymine-requiring mutants and of mutants that are temperature-sensitive for the initiation of

chromosome replication (Mendelson & Gross, 1967) made it possible to test this assumption by fairly simple experiments. The results show that there is a sensitive period during the division cycle, when sporulation can be induced, but this period does not coincide with the completion of chromosome replication. Instead, it seems that if cells are to be induced to sporulate they must be transferred to conditions of poor growth while chromosome replication is still proceeding. If the transfer is effected after replication has been completed in a rich medium the chromosomes are in the wrong position and sporulation does not occur.

MATERIALS AND METHODS

Organisms. *B. subtilis* 168 (Marburg) is an auxotroph requiring tryptophan or indole. It sporulates normally and is referred to as the wild-type *B. subtilis*; (thy⁻A) is a thymine-requiring mutant derived from *B. subtilis* 168. We also used *B. subtilis* ts-134, which has a requirement for thymine and is temperature-sensitive for initiation of chromosome replication, i.e. at 45°C any chromosome replication that has begun will be completed but no fresh rounds of replication will be initiated. At permissive temperatures (35°C or lower) replication and growth proceed normally (Mendelson & Gross, 1967).

Culture and procedure for inducing sporulation. Unless stated otherwise, growth and sporulation experiments were performed at 35°C. The bacteria were grown in a medium containing hydrolysed casein and inorganic ions together with L-tryptophan (20 µg/ml). This is termed 'rich medium'. When necessary it was supplemented with thymidine (20 µg/ml). When the bacterial density reached 0.25 mg dry wt./ml the culture was centrifuged and sporulation was initiated by transferring the cells to the same volume of resuspension medium sometimes termed 'poor medium'. This consisted of glutamate, tryptophan and inorganic ions with a high concentration of Mg²⁺ (Sterlini & Mandelstam, 1969). Where indicated it was supplemented with thymidine (20 µg/ml). Incubation with shaking at 35°C in this medium generally gave a yield of about 80% refractile spores in 8–10 h with the wild-type. For some unknown reason the spore yield with ts-134 was somewhat variable and always much lower (15–30%). Strain 10-ts-B19 was even less satisfactory in its sporulation.

Estimation of spore incidence. Cells containing refractile spores were counted in the phase-contrast microscope and values are expressed as a percentage of the total cell population.

Bacterial growth. This was measured spectrophotometrically by using a calibration curve relating E_{600} to bacterial dry wt.

Electron microscopy. Samples of culture were taken and sections prepared as described by Kay & Warren (1968). Cells were assessed morphologically only if it was apparent that they had been sectioned longitudinally and close enough to the equatorial plane to give a representative view of the whole cell.

Determination of protein, DNA and RNA. Samples (6 ml) were taken from resuspension cultures and analysed by standard methods. For details see Waites *et al.* (1970).

Alkaline phosphatase. The enzyme was assayed by a modification (Sterlini & Mandelstam, 1969) of the method used by Torriani (1960). One enzyme unit hydrolyses one nmol of *p*-nitrophenylphosphate/min.

RESULTS

Synthesis of protein, DNA and RNA during sporulation in the wild-type. To initiate sporulation wild-type bacteria were transferred from rich medium to resuspension medium as described in the Materials and Methods section. At zero time (t_0) and at hourly intervals samples were taken for analysis. Refractile spores in countable numbers (>1%) had usually appeared by about 4 h and then increased rapidly. The protein and dry wt. amounts increased in parallel and generally doubled in the first 4–5 h (Fig. 1). The total RNA amount remained constant or tended to decrease slowly (Fig. 1). The amount of DNA increased by 40–45% in some experiments, a value that is close to the theoretical increase that would be expected if each chromosome had a single replication fork and if DNA synthesis ceased when replication had been completed (Maaløe & Hanawalt, 1961). More commonly the amount of DNA increased by

Fig. 1. Synthesis of protein and nucleic acids during sporulation. The figures show the changes that occur after wild-type bacteria have been transferred from a rich growth medium to a resuspension medium to induce sporulation: (a) ○, protein; ●, dry weight. (b) △, Total RNA; ▲, DNA. Refractile spores were apparent after 4 h.

110–150% (Fig. 1) indicating either the existence of multiple replication forks or possibly the initiation of new rounds of replication. No reason could be found for these differences in the amount of DNA synthesized and they did not have any apparent effect on the subsequent development of spores.

Requirement for DNA synthesis during sporulation. To determine to what extent DNA synthesis after resuspension was obligatory for sporulation we made use of the thymine-requiring mutant (thy⁻A). After growth in rich medium with thymidine the organisms were transferred to resuspension medium (100 ml) also containing thymidine. At t_0 and at intervals up to 90 min, portions (20 ml) of the culture were removed and rapidly centrifuged, then the supernatant was poured off and the tubes allowed to drain thoroughly. The cell pellet was resuspended to the same density in fresh resuspension medium, this time without thymidine, and the incubation was continued. A control portion was centrifuged and resuspended

Fig. 2. Effect of thymidine deprivation on the development of refractile spores. To induce sporulation thymine-requiring cells (thy⁻A) were transferred to resuspension medium containing thymidine. At intervals portions of the culture were centrifuged and the cells incubated further after resuspension in medium containing no thymidine. The time at which thymidine deprivation occurred is indicated as follows: ○, 90 min; △, 40 min; ▲, 20 min; ●, control (thymidine present throughout). In the portion in which thymidine was removed at zero time the final incidence of spores was <2%. This curve is not shown.

with thymidine to ensure that the manipulations were not affecting the spore yield.

When thymidine was present throughout, refractile spores began to appear at about 4.5 h; at 8 h the culture contained 60% spores and the values were still increasing (Fig. 2). In the cells that were deprived of thymidine at 40 min and at 20 min the final spore yield was decreased to about 20% and 5% respectively, while in the culture deprived at zero time the spore count was generally <2%

(Fig. 2). Some day-to-day variability in the t_0 samples may have been caused by differences in the carry-over of thymidine. Although washing the cells might have eliminated this variability and reduced the t_0 values still further it was not done because experience had shown that this procedure had an adverse and not very reproducible effect on sporulation.

Effect of thymidine-deprivation on early steps in the sporulation sequence. Prevention of DNA synthesis largely prevented the appearance of refractility, which is a 4 h event in spore development, but DNA synthesis was not shown to be necessary for any of the earlier stages of sporulation. We therefore decided to use the presence of alkaline phosphatase as a marker event. This enzyme is produced about 2 h after resuspension and its appearance is correlated with stage II in spore development (see Waites *et al.* 1970).

The experiment was done as before except that thymidine was removed at t_0 and at 60 min. In these two cultures the amount of alkaline phosphatase and the number of refractile spores were decreased proportionately. Thus in the culture deprived at 60 min the amount of enzyme and the number of cells with refractile bodies were both decreased by about 50% relative to the values for the control culture in which thymidine was present continuously (Fig. 3). In the culture deprived at t_0 alkaline phosphatase and the incidence of refractile spores were proportionately decreased to very low values.

Electron microscopy was performed on a sample taken at 2 h and at 4 h from a culture deprived of thymidine at t_0 . Virtually all the cells retained the appearance of normal vegetative cells with distinct and compact chromosomes, with no evidence of development of spore septa except in the very small proportion of cells that were already known from the refractility measurements to be undergoing sporulation. The cells resembled those shown in Plate 1(a) and are not illustrated.

Relationship between sporulation and the DNA replication cycle. The experiments described above were consistent with the view that induction of sporulation could not occur if the chromosomes were in a partly replicated state. It seemed reasonable to assume as a working hypothesis that induction could take place when the replication had gone to completion and that this might be the stage of the division cycle at which cells were sensitive to induction. The properties of the temperature-sensitive mutants, ts-134 and 10-ts-B19, made them suitable for experiments to test this.

Preliminary experiments were done to determine the amount of DNA that could be synthesized at the restrictive temperature. The organisms were grown at 35°C in hydrolysed casein medium (100 ml) supplemented with thymidine. When the culture

Fig. 3. Comparison of effects of thymidine deprivation on early (alkaline phosphatase) and later (refractility) steps in sporulation. Thymine-requiring cells (thy⁻A) were transferred to resuspension medium with thymidine and subsequently subjected to thymidine deprivation as described in the legend to Fig. 2. Samples were taken periodically for estimation of alkaline phosphatase (a) and refractility counts (b). Times of thymidine deprivation were: ○, 60 min; △, 0 min; ●, control (thymidine present throughout).

density had reached 0.1–0.15 mg/ml the flask was transferred to a water-bath at 45°C and incubation with shaking was continued for 2–3 h. With 10-ts-B19 the increase in DNA amount at the high temperature was usually 100–150%, i.e. the same as in the wild-type and rather more than the value of 80% found by Gross, Karamata & Hempstead (1968). The subsequent sporulation behaviour was qualitatively similar to that of ts-134 (see below) but very variable from one experiment to another and the spore incidence was generally lower. It was therefore not used as a routine.

When similar experiments were done with ts-134 the increase was usually 40–50% and most of it occurred in the first 30–45 min (see Fig. 4 below). The results were similar to those reported by Mendelson & Gross (1967) although the medium they used was richer. When a portion of the culture taken at 45 min was transferred back to 35°C, growth was resumed almost immediately and the normal doubling time (49–50 min) had been restored in well under 30 min, showing that the cells had not been damaged by the heat treatment. From these experiments it seemed that 45 min at the high temperature would be enough to allow most, if not all, of the cells to complete their DNA synthesis while leaving the majority of them viable.

To determine the sporulative capacity of cells in which chromosome replication had occurred cul-

tures in growth medium supplemented with thymidine were shaken at 45°C for 45 min. During this period samples were taken for analysis of DNA to check that the expected amount of synthesis had taken place. The cultures were centrifuged and the cells transferred to resuspension medium with or without thymidine and shaken at 35°C. The assumption underlying the experiment was that because the requisite DNA synthesis had largely taken place by this time, most of the cells would be in the inducible state and would sporulate whether they were supplied with thymidine or not. However, this assumption was completely contradicted by the results of the spore counts made in the ensuing 9 h. These showed that even with thymidine present sporulation was poor (2% or less) and that without thymidine it was even worse (< 0.2%).

Effect of the type of growth medium during chromosome replication on subsequent sporulation. Because the failure of cells to sporulate in the above experiment was apparently not attributable to cell damage resulting from the heat treatment, alternative explanations were considered. The simplest of these was that the cells had to be subjected to the semi-starvation produced in the resuspension medium before the chromosome replication had been completed and that once replication had occurred in a rich growth medium,

EXPLANATION OF PLATE I

(a) A typical cell of *B. subtilis* ts-134 undergoing vegetative growth. A division septum is forming half-way along the cell and the chromosomes in both halves of the cell appear as compact white areas. The bar represents $1\mu\text{m}$. (b) A pair of cells of *B. subtilis* ts-134 that have completed DNA synthesis in a rich medium at 45°C (see text). The chromosomes retain a compact appearance. The bar represents $1\mu\text{m}$. (c) A cell of *B. subtilis* ts-134 that has completed DNA synthesis in a poor medium at 45°C (see text). The chromosomes (there are probably two) are elongated into a filament stretching along the axis of the cell. This appearance is typical of stage I of sporulation. The bar represents $1\mu\text{m}$.

the cells would be 'primed' for growth but not for sporulation.

Accordingly, a culture of ts-134 was grown at 35°C in rich medium (100ml) supplemented with thymidine; the suspension was centrifuged and half of the cells suspended in more of the same growth medium (100ml) and the other half was put into resuspension medium (50ml) also supplemented with thymidine (see legend to Fig. 4). Both cultures were shaken at 45°C for 45 min. In a typical experiment the dry wt. of cells in the rich medium increased exponentially during this period from 0.10 to 0.29 mg/ml while in the poor medium there was a linear increase from 0.21 to 0.29 mg/ml. Although the growth was different, the DNA in both cultures had increased at about the same rate and to about the same extent (Fig. 4). The two cultures were centrifuged and the cells transferred to resuspension medium. Each suspension was then sub-divided into two portions, one of which was supplemented with thymidine. All the suspensions, which at this stage contained 0.27–0.29 mg dry wt. of cells/ml, were shaken at 35°C to allow sporulation to occur. A control experiment was done in which cells that had not been subjected to the high temperature were transferred directly from growth medium at 35°C to resuspension medium with thymidine and then incubated at the same temperature. The incidence of spores in all cultures was determined at the end of 9 h.

As before, completion of chromosome replication in the rich growth medium produced cells that produced few spores in the absence of thymidine and not many in its presence (Table 1, cf. lines *a* and *c*). However, cells that had completed replication in the poor medium sporulated to the extent of 10–12% after 9 h whether thymidine was present or not (Table 1, lines *b* and *d*) and the spore incidence was almost certainly still increasing, though further readings were not taken. This compares well with the value obtained with the control culture that was not exposed to the high temperature.

Electron-microscopy of cells that had completed chromosome replication in rich and in poor medium. The above result supported the view that if sporulation was to be initiated the cells had to be stimulated by transferring them to the poor medium before replication was complete, and that this was affecting the cells in some radical way that might be morphologically apparent even though the total amounts of DNA synthesised in the two media were very similar.

Cells in the exponential growth phase at 35°C in rich medium with thymidine were either transferred to more of the same medium or to poor medium also with thymidine and incubated further at the high temperature (45°C). The cultures were sampled at the times of the transfer and again after

Fig. 4. Increase in DNA amount in temperature-sensitive mutant ts-134 at the restrictive temperature. Mutant ts-134 (temperature-sensitive for initiation of chromosome replication) previously grown at 35°C was suspended either in rich medium (○) or in poor medium (●), both containing thymidine. The suspensions were incubated at 45°C and sampled periodically. The concentrations of DNA at zero time were 2.0 and 4.0 μ g/ml culture respectively. (The cells in the rich medium were suspended at half the density to allow for the fact that their growth in the ensuing incubation would be much greater: see the text.)

Table 1. *Effect of chromosome replication in rich and poor media on subsequent sporulation*

Mutant ts-134 (thymine-requiring and temperature-sensitive for initiation of chromosome replication) was incubated with thymidine in either rich or poor medium at 45°C for 45 min to allow DNA synthesis to be completed. The cells were then transferred to poor medium and spore incidence was determined after incubation at 35°C for 9 h. In a control experiment, cells were transferred straight from the rich medium with thymidine at 35°C to the poor medium, also with thymidine, at the same temperature. In these cells the incidence of refractile spores was also 12% at 9 h.

Medium during first incubation	Presence of thymidine in second incubation (poor medium)	Spores (% at 9 h)
(a) Rich	—	< 0.1
(b) Poor	—	10.0
(c) Rich	+	< 3.0
(d) Poor	+	12.0

35 min, i.e. at the time when the period of rapid DNA synthesis was approaching its end.

Sections of the initial sample showed cells

having the appearance that would be expected of normal cells growing in a full medium. They had chromosomes that appeared as compact and well-separated areas resembling those illustrated in Plate 1(a). After 35 min in the rich medium at high temperature the mesosomes had become more prominent and the cells themselves had elongated. Nevertheless, the chromosomes retained the separate and compact appearance typical of vegetative cells (Plate 1b). By contrast, the cells that had been at the high temperature in poor medium for the same time had chromatin material appearing as an extended band down most of the length of the cell (Plate 1c). This axial filament is found in cells that have been initiated to sporulation and is the defining morphological characteristic of stage I in spore development (Murrell, 1967).

An additional control experiment was done. Samples for electron microscopy were taken of cells that had been transferred straight from full growth medium with thymidine to resuspension medium without thymidine. In this experiment there was no intervening period of treatment at high temperature. At the end of 4 h of thymine-deprivation these cells retained the chromosome appearance and distribution of normal vegetative bacteria. The cells were not discernibly different from those in Plate 1(a) and are therefore not shown.

DISCUSSION

When wild-type cells are transferred from rich medium to resuspension medium to induce sporulation, the total DNA sometimes increases by an amount that is consistent with the completion of single-forked chromosomes. More frequently, under apparently identical conditions, the amount of the increase is 100% or more, which would be consistent with multiple forks (Sueoka & Yoshikawa, 1965). Similar variability is apparent in the behaviour of the two temperature-sensitive mutants. We can only conclude that the growth conditions in our experiments were critical and that multiple forks were produced in some experiments but not in others. Nevertheless, the experiments on thymidine deprivation of cells showed clearly that the increase in DNA amount that occurs when they are transferred to resuspension medium is essential for sporulation. If the bacteria are prevented from performing this synthesis the majority of them fail to form refractile spores and do not even perform early steps of the process such as forming a spore septum or synthesizing alkaline phosphatase. Electron microscopy shows that, except in the very small percentage of cells that sporulate under these conditions, the bacterial chromosomes remain separate and compact as they are in the normal vegetative cell.

Initially these experiments appeared to support the view expressed in the Introduction that sporulation could be initiated only after the chromosomes had finished replication. This assumption is reasonable in view of the experiments of Oishi *et al.* (1964), which implied that the spores of *B. subtilis* contained chromosomes that were without replication forks. However, the results in Table 1 indicate that if the cells have completed DNA synthesis in a rich medium it is too late to induce sporulation. These cells, when deprived of thymidine, form virtually no spores (<1.0%, Table 1), whereas 10% of those that had completed DNA synthesis in poor medium had formed spores after 9 h. The experiments with these temperature-sensitive mutants thus point to the conclusion that if spore formation is to be initiated the transfer of cells to poor medium must take place while replication is proceeding. Electron micrographs of cells from these cultures indicate why replication in a rich medium may largely preclude subsequent sporulation. The photographs (Plate 1b and 1c) show that the nature of the growth medium apparently determines the position that the chromosomes assume and also determines whether they are compact spherical 'nucleoids' or whether they are spread out as a diffuse filament along the cell. Thus, at 35 min, i.e. before DNA synthesis has finished, the chromosomes are positioned and the cells are 'primed', for growth if the replication occurred in a rich medium, or for sporulation if it occurred in a poor one. In the latter case the cells are in stage I of spore development by the time DNA synthesis has ceased. This is so even though the net synthesis of DNA in the two media is the same.

The two 'primed' states differ in the extent to which they are reversible. The cells that are primed for growth are unable to sporulate and if provided with thymidine, do so very poorly. However, the cells that are primed for sporulation can revert readily to vegetative growth. Thus, when such cells were returned to a rich growth medium they were able to return to their normal rate of growth with very little lag. This would be expected since Fréhel & Ryter (1969) observed that cells that are even further advanced in sporulation can return to the vegetative state.

It remains to be explained why, at some particular period during the replication process, the distribution of the chromosomes in the cytoplasm depends on whether the cells have, or have not, been exposed to the step-down conditions of the sporulation medium. We know very little about the metabolic consequences of step-down situations and even less about how the alterations in the amount and types of metabolites determine the shape and position of the chromosomes. If the position of the

chromosome is determined by that of the mesosome to which it is presumably attached we must assume that a change in the amount or type of some metabolic constituent (e.g. ATP or a lipid component) causes the newly forming mesosome to form at the pole of the cell instead of nearer the centre and that this in turn primes the cell for sporulation rather than for growth. Whether the primary effect of step-down conditions is on the mesosome or whether some other part of the cell structure is responsible for the position of the chromosome (see Highton, 1970), it nevertheless appears that spore induction is compatible only with a certain position of the chromosomes and that this must have been imposed while they were being formed. A simple model for spore induction based on these considerations has been proposed by Dawes, Kay & Mandelstam (1971).

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REFERENCES

- Dawes, I. W., Kay, D. & Mandelstam, J. (1971). *Nature, Lond.*, **230**, 567.
Dawes, I. W. & Mandelstam, J. (1970). *J. Bact.* **103**, 529.
Fréhel, C. & Ryter, A. (1969). *Annls Inst. Pasteur, Paris*, **117**, 297.
Gross, J. D., Karamata, D. & Hempstead, P. G. (1968). *Cold Spring Harb. Symp. quant. Biol.* **33**, 307.
Highton, P. J. (1970). *J. Ultrastruct. Res.* **31**, 260.
Kay, D. & Warren, S. C. (1968). *Biochem. J.* **109**, 819.
Maaløe, O. & Hanawalt, P. C. (1961). *J. molec. Biol.* **3**, 144.
Mendelson, N. H. & Gross, J. D. (1967). *J. Bact.* **94**, 1603.
Murrell, W. G. (1967). *Adv. microb. Physiol.* **1**, 133.
Oishi, M., Yoshikawa, H. & Sueoka, N. (1964). *Nature, Lond.*, **204**, 1069.
Ryter, A. & Aubert, J.-P. (1969). *Annls Inst. Pasteur, Paris*, **117**, 601.
Sterlini, J. M. & Mandelstam, J. (1969). *Biochem. J.* **113**, 29.
Sueoka, N. & Yoshikawa, H. (1965). *Genetics, Princeton*, **52**, 747.
Torriani, A. (1960). *Biochim. biophys. Acta*, **38**, 460.
Waites, W. M., Kay, D., Dawes, I. W., Wood, D. A., Warren, S. C. & Mandelstam, J. (1970). *Biochem. J.* **118**, 667.