Resporulation of Outgrowing *Bacillus subtilis* Spores

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Germinated spores of *Bacillus subtilis* were incubated in outgrowth medium and tested periodically for capacity to sporulate when suspended in sporulation medium. Concurrent measurements were made of deoxyribonucleic acid (DNA) content and numbers of cell division septa and nucleoids. Sporulation potential was shown to reach a peak at about 110 min, at which time the chromosomes are probably well into the second round of replication. Experiments with nalidixic acid show that sporulation potential can be generated in the outgrowth medium even when DNA synthesis is largely prevented. Further experiments show that nalidixic acid apparently does not prevent the formation of DNA initiation complexes, which can subsequently function after resuspension in the sporulation medium. The results support those previously obtained with a temperature-sensitive DNA mutant which indicated that sporulation could only be induced at a specific stage of chromosome replication, and then only if the cells are in a state of nutritional "step-down."

There is some evidence that suggests that the initiation of bacterial sporulation occurs at a specific point in the growth cycle provided that the cells are nutritionally in a "step-down" environment at the time. Dawes and Mandelstam (6) formulated this hypothesis to explain the results of their experiments on the sporulation of *Bacillus subtilis* in chemostat cultures. Further support came from the work of Dworkin et al. (7) and Mandelstam and Higgs (14), who used cells synchronized with respect to chromosome replication. This work pointed to a link between induction of sporulation and a specific stage in the deoxyribonucleic acid (DNA) replication cycle. In these experiments a mutant was used that was temperature sensitive for the initiation of chromosome replication, and synchrony was imposed by first "lining up" the chromosomes at the restrictive temperature.

The use of temperature-sensitive mutants to impose synchrony might produce artefacts, and it would be useful to have an alternative system in which to examine the possible relationships between the induction of sporulation and chromosome replication. One obvious possibility was to study the resporulation of outgrowing *B. subtilis* spores. This system seemed promising because it had already been shown that there was synchrony of chromosome replication during outgrowth (17). In addition, Mychajlonka and Slepecky (16) had demonstrated that spores of *Bacillus megaterium* could be induced to resporulate only during a limited period of their outgrowth.

Although the primary object of our work was to study the interrelationship of chromosome replication and the induction of sporulation, the experimental conditions were those that in other bacilli lead to "microcycle" sporulation. This was defined by Vinter and Slepecky (22) as the "direct transition of outgrowing bacterial spores to new sporangia without intermediate cell division." Since in our studies we could not demonstrate that resporulation in *B. subtilis* does occur without an intervening cell division, we have avoided the use of the term "microcycle" sporulation. Nevertheless, these experiments permit one to compare resporulation of outgrowing spores of *B. subtilis* with that of outgrowing spores of *B. megaterium* and *Bacillus cereus*. Spores of the latter species contain amounts of DNA equivalent to two chromosome complements and each spore gives rise to a primary cell that contains two nuclear bodies. In contrast, spores of *B. subtilis* contain only one chromosome equivalent and produce primary cells that contain a single nuclear body (9, 11).

The results to be described support the hypothesis that induction of sporulation is linked to a specific stage in the cell cycle. In addition, we have found differences between the resporulation behavior of *B. subtilis* and that of the other bacilli, which may result from differences in the initial chromosome contents of their spores.
MATERIALS AND METHODS

Preparation of spores. Spores of B. subtilis 168 trpC2 were obtained after growth and sporulation of cells in the resuspension medium of Sterlini and Mandelstam (21) supplemented with glucose (2 g/liter). The spores were purified using the method of Bonsen et al. (1) and then centrifuged through 50% Urografin (Schering Chemicals Ltd.) to remove any residual germinated spores (20). Spores were stored either as a lyophilized powder or as a suspension (2 × 10^9 to 4 × 10^9/ml) in distilled water.

Heat activation. Immediately before use, spore suspensions (2 × 10^9 to 4 × 10^9/ml in distilled water) were incubated at 70°C for 30 min.

Germination. Germination took place during incubation of spores (2 × 10^9 to 4 × 10^9/ml) at 37°C for 90 min in a medium containing (per liter): L-alanine, 1.0 g; NaCl, 0.59 g; Na2SO4, 0.1 g; KH2PO4, 0.07 g; Tween 80, 0.1 g. More than 95% of the spores germinated during this incubation.

Outgrowth. Germinated spores were centrifuged, suspended in an equal volume of brain heart infusion broth (Difco) containing 5 g of yeast extract (Difco) per liter, and incubated at 37°C. During the first few minutes the incidence of nongerminated spores fell to less than 1%.

Resporulation. Outgrowing spores were incubated at 37°C in a nitrogen-free sporulation medium modified as follows from the resuspension medium of Sterlini and Mandelstam (21). NH4Cl and NH4NO3 were replaced by NaCl, 0.59 g/liter; sodium glutamate was replaced by sodium lactate, 2.6 g/liter. These changes reduced growth during resporulation. After outgrowth in the presence of nalidixic acid, resuspended samples were osmotically sensitive and lysis was minimized by the use of sodium lactate at 30 g/liter. Sporulation was not affected by the higher concentration of sodium lactate.

Cell counts. Viable counts were made by plating cultures on nutrient agar (Difco) after dilution with sporulation medium. For measurement of numbers of heat-resistant spores, cultures were heated at 80°C for 15 min and then plated.

Septa. Septa were counted by phase-contrast microscopy after plasmolysis of cells in CsCl (7).

Nuclear bodies. Nuclear bodies were observed after Giemsa staining as described by Sargent (19).

Cell growth. Cell growth was followed by measurements of extinction at 600 nm (E_600) of suitably diluted culture samples.

DNA estimation. DNA was estimated by using the method of Burton (3). It would have been more convenient and more accurate to measure DNA synthesis by incorporation of radioactively labeled thymidine. However, the spores of thymine-requiring mutants appear to have uncompleted chromosomes and, hence, to have extra replication forks during germination (8, 10). Although a different interpretation has been placed on the findings (4), there is no doubt that the chromosomes of the thymine mutants are in a different state from those of the wild type, and this precluded the use of a thymine-requiring strain in these experiments.

Inhibitors. DNA synthesis was prevented by the addition of 20 μg of nalidixic acid (NAL) per ml (Calbiochem) or 50 μg of 6-(p-hydroxyphenylazo)uracil (HPUra) per ml (a gift from B. Langley of Imperial Chemical Industries Ltd.). Protein synthesis was prevented by the addition of 100 μg of chloramphenicol per ml (Sigma).

RESULTS

Chromosome replication and septation during outgrowth. We wished to know the times at which successive rounds of DNA replication and cell division occurred during outgrowth. Accordingly, germinated spores were suspended in outgrowth medium and samples were removed at intervals for measurements of E_600 and DNA content. Nuclear and cell division were followed microscopically.

Under our conditions, spore elongation began after 50 min. By about 80 min the cells formed short rods. These grew into long filaments, some of which were almost straight, whereas others were quite twisted. The first nuclear division began at about 90 min and was half complete at about 100 min. Further nuclear divisions occurred at about 20-min intervals (Fig. 1A). Each wave of nuclear division was followed after about 25 min by a wave of septation.

Since the first nuclear division occurred at about 100 min, it is apparent that the first round of DNA replication must have been initiated considerably earlier. The data shown in Fig. 1B indicate that DNA synthesis started about 50 min after the beginning of outgrowth. This accords with earlier reports that the time taken to replicate the chromosome in B. subtilis is, in fact, about 50 min (14, 20).

Resporulation potential during outgrowth. Samples (5 ml) were removed from an outgrowing culture and centrifuged. The bacterial pellets were then washed with, and suspended in, sporulation medium. After incubation for a further 20 h, the number of heat-resistant spores in each sample was determined. The potential to resporulate was confined to a restricted period during outgrowth; it increased at about 70 to 80 min, reached a maximum at about 110 min, and declined thereafter (Fig. 2A). The time at which maximum resporulation potential occurred corresponds to the time when the first nuclear division was almost completed and septation was beginning (Fig. 1A).

Effect of medium exchange on sporulation potential. The above results were consistent with a linkage between resporulation potential and degree of outgrowth. However, the possibility had to be considered that the changes in sporulation potential could have resulted from
changes in the outgrowth medium. To eliminate this possibility, the following experiment was carried out. Germinated spores were incubated in outgrowth medium for 100 min. The cells were then resuspended to the same density in a fresh batch of outgrowth medium. The partially exhausted medium was kept and used to establish outgrowth of a fresh batch of spores. Both cultures were tested as before for their ability to resporulate, and in both the peak occurred at about 110 min after the initiation of outgrowth.

Effect of prevention of DNA synthesis in the outgrowth medium. The following experiments were done to determine whether DNA synthesis in the outgrowth medium was an essential prerequisite for the development of sporulation potential. Germinated spores were outgrown in the presence of NAL and the culture was assayed periodically for total DNA.
There was very little DNA synthesis (Fig. 1B): the net increase was 40% in 180 min. (In a control culture, without NAL, DNA increased at an accelerating rate after a lag of about 50 min.)

Samples (5 ml) were taken periodically from the culture with NAL and suspended in sporulation medium. The number of heat-resistant spores in each sample was determined after 20 h. In spite of the fact that NAL had largely prevented synthesis of DNA, it did not prevent the development of sporulation potential (Fig. 2B). The potential, having once developed, did not then decline. In some experiments it reached a plateau value at 140 min; in others it continued to increase throughout the period of outgrowth. Both types of result are illustrated.

Generation of replication forks in outgrowth medium during inhibition of DNA synthesis. The hypothesis under test is that sporulation can be initiated only during a restricted period in the DNA replication cycle and then only if the cells are in a step-down medium at the time. If this is correct, it implies that the previous experiment should be interpreted as follows. When NAL was present during outgrowth and DNA synthesis was largely suppressed, the cells were nevertheless able to generate new DNA replication sites, which could then have become functional when the cells were subsequently transferred to sporulation medium.

To test this, germinated spores were incubated in outgrowth medium in the presence of NAL. Periodically, samples were removed, the cells were washed and incubated for 90 min in outgrowth medium without NAL but with chloramphenicol, and the total DNA was then measured. This procedure allowed the completion of any rounds of replication that had been initiated in the presence of NAL but prevented the initiation of new rounds (23).

In the control (without NAL) the first replication fork was initiated at about 35 min (Fig. 3), which is consistent with the fact that net synthesis of DNA was measurable 10 to 15 min later (cf. Fig. 1B). After outgrowth for 115 min, sufficient replication forks had been generated to result in a 15-fold increase in final DNA.

When NAL was present there was little net DNA synthesis in the outgrowth medium (see Fig. 1B). However, initiation sites were nevertheless generated, starting at about 40 min (Fig. 3), and by the end of the experiment the cells had accumulated the potential for a fourfold increase in final DNA.

Cell growth and DNA synthesis in sporulation medium. Earlier work had shown that when cells from an exponential culture were transferred to resuspension medium, both growth and DNA synthesis occurred (5). Also, if the DNA synthesis was prevented, sporulation failed to occur. Experiments were therefore done to determine whether these findings also applied in the conditions of resporulation.

Samples from an outgrowing culture were taken at 100 and 160 min and transferred to
sporulation medium. During the next 7 h measurements were made of DNA content and turbidity. In the sample suspended at 100 min, the \( E_{660} \) value remained constant after a slight initial increase (Fig. 4A). The DNA content of the culture increased by about 30% between 2 and 4 h after resuspension and then remained constant (Fig. 4A). Heat-resistant spores began to appear in the culture 11 h after resuspension. In the 160-min sample, the extinction of the culture decreased slowly throughout the experiment, and the DNA content, after an initial increase, also declined (Fig. 4B). By about 8 h considerable amounts of cell debris were visible in the phase-contrast microscope, and many of the residual cells were obviously lysing. These cells did not sporulate.

To determine the effect of preventing DNA synthesis, spores were outgrown for 100 min and then suspended in sporulation medium in the presence of HPURA (a drug that specifically inhibits DNA replication in B. subtilis [2]). In the presence of HPURA sporulation was reduced to 1% of the control. Similar results were obtained when a thymine-requiring mutant (Thy–A) was incubated in sporulation medium with and without thymidine.

NAL was unsuitable for inhibiting DNA synthesis in the sporulation medium, because sporulating cells apparently escape from the inhibitory effect of NAL. (The reasons for this have still to be investigated.)

**DISCUSSION**

Previous work in this laboratory suggested that cells of B. subtilis can be induced to sporulate only during a restricted part of the DNA replication cycle (6, 7, 13–15). Furthermore, the successful termination of existing rounds of DNA replication was a necessary prerequisite for sporulation (15). Considering these studies together, one can infer the following sequence of events when vegetative cells are transferred from a growth medium to a resuspension medium.

(i) The chromosome replication in each cell will come to an end and be followed at the appropriate interval by cell division (5).

(ii) The subsequent behavior of the resulting daughter cells will be determined by the stage of DNA replication at which the step-down was made. If it was after the first 15 min (approximately) of chromosome replication, the cells will require another round of vegetative growth before they can sporulate (5) and new DNA replications will be initiated. If, however, the step-down was made during the first 15 min (approximately), two signals will be generated. One of these is an instruction to the daughter cells not to initiate any new chromosome replications; the other is an instruction to begin sporulation.

In our experiments, outgrowing spores transferred to the step-down condition before the beginning of the first replication, i.e., before about 45 min, have only one chromosome each, and this will not yet have begun to replicate. Such cells clearly could not be expected to sporulate.

Those cells transferred after the beginning of the first replication cycle, i.e., between 45 and 75 min (approximately), will have only one replicating chromosome and will be able to carry out only the instruction not to initiate further rounds of DNA synthesis. Each cell will complete chromosome replication and divide into two daughter cells, each of which will still contain only a single chromosome. Again, such cells could not be expected to sporulate.
This reasoning may explain the differences in resporulation behavior of *B. subtilis* in comparison with that of the microcycle sporulation in *B. cereus* and *B. megaterium* described by Vinter and Slepecky (22) and Holmes and Levinson (12). In the two latter species, each spore starts germination and outgrowth with two chromosomes (9, 11) and therefore already has double the nuclear capacity of *B. subtilis*. The spores of the large bacilli can apparently undergo microcycle sporulation without cell division (12, 22), whereas in *B. subtilis* there has to be an extra DNA replication cycle accompanied by cell division.

Outgrowing spores transferred after about 75 min have initiated the second or third rounds of DNA replication, and there is probably by now some loss of synchrony. Some cells in the population will have been transferred to the step-down medium during the early stages of the second or third rounds of replication and they will divide to give two or four daughter cells, each of which will contain two chromosomes and will therefore sporulate. Cells in this class would therefore be expected to show an increase in sporulation potential up to 110 min.

We now have to consider the cells transferred later, e.g., after 150 min in outgrowth medium. These cells should resemble those in the previous class; many individuals should have early replication forks, which should traverse the critical point on the chromosome while the cells are in the step-down medium. These cells should, therefore, have a large sporulation po-
tential, which ought to be expressed. They should, in fact, resemble ordinary vegetative cells, the majority of which sporulate after resuspension. However, they do not, and this requires explanation. At present we have only some preliminary evidence that indicates that cells at this stage do not survive resuspension in the sporulation medium (Fig. 4B). Further work is required to determine how long growth in the broth has to be continued before the cells begin to behave like those of a "normal" vegetative culture.

 Whereas NAL greatly reduces DNA synthesis in the outgrowth medium, it does not prevent development of the sporulation potential. In the presence of the drug, a number of new initiation complexes for chromosome replication are generated. These can become functional when the NAL is removed and the cells are transferred to sporulation medium. The fact that sporulation potential develops in NAL supports the assumption that what is critical is that the replication fork should pass through the sensitive point of the chromosome while the cells are in a step-down condition. If the replication fork is prevented from doing this by inhibiting DNA synthesis in the sporulation medium by using HPUra or thymine starvation, spor formation is inhibited. This, again, is what would be expected from the results obtained with cells from vegetative cultures (15).

 Finally, we point out that the ability to induce differentiation during a restricted period in the cell cycle is not limited to sporeforming bacteria. Analogous instances have been reported in many types of eucaryotic cells (18).

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LITERATURE CITED


