SHORT COMMUNICATION

Dissociation of an Early Event in Sporulation from Chromosome Replication in *Bacillus subtilis*

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Synchronized populations of *Bacillus subtilis* are maximally inducible for sporulation about 15 min after chromosome replication has started. However, the induction of serine protease, one of the earliest marker events in sporulation, is not related to the state of chromosome replication.

INTRODUCTION

Sporulation in *Bacillus subtilis* is readily induced by transferring the cells from a rich medium to a poor one, and there is a relationship between sporulation potential and the stage of chromosome replication at the time of the transfer to the 'step-down' medium. This relationship is shown by the following facts. (1) If a temperature-sensitive mutant is used to synchronize chromosome replication there is a critical point about 15 min after the beginning of a new replication at the permissive temperature at which the step-down stimulus is effective. After this the cells quickly lose their ability to respond, and sporulation cannot then be induced until the next round of replication (Mandelstam & Higgs, 1974). A peak in sporulation potential can also be demonstrated in synchronized populations obtained from outgrowing spores (Keynan *et al.*, 1976). (2) When hydroxyphenylazouracil (HPUra), a specific inhibitor of DNA synthesis, is added to portions of an unsynchronized culture at intervals after sporulation has been induced, the population begins to escape from the inhibitory effect of the drug at a time corresponding to that at which the first cells complete their chromosome replication (Dunn *et al.*, 1978). The conclusion that sporulation cannot occur without chromosome completion was confirmed by gene-frequency analysis and it fits well with the earlier genetical studies of Oishi *et al.* (1964). These showed that spores of *B. subtilis* apparently contained only complete chromosomes. In addition, the experiments with HPUra provided an independent estimate of the critical point. They showed that the cells begin to escape about 35 to 40 min after transfer to the step-down medium. Since the chromosome replication time is about 55 min, the implication was that the first cells to escape had completed the first 15 to 20 min of their chromosome replication at the time the step-down was imposed.

In all these experiments, sporulation potential was measured by the production of either phase-bright or heat-resistant spores, i.e. by very late sporulation events. Early events in sporulation, such as the production of protease, have not been examined in relation to the critical point, although Young & Jeffs (1978) used partially inhibitory concentrations of HPUra to slow down DNA synthesis and showed that protease formation could begin before chromosome replication was complete.

The present experiments were done to establish whether the onset of protease synthesis is related to the critical point. The results show that the enzyme is induced irrespective of the state of chromosome replication at the time of step-down.

METHODS

Organism. *Bacillus subtilis* ts-134 (168 *trpC2 thyA thyB ts-dnaB*) was used throughout (Mendelson & Gross, 1967). It is temperature-sensitive for the initiation of chromosome replication. At the permissive temperature (35 °C) replication is normal whereas at 45 °C replications in progress are completed but no new ones are initiated (Gross *et al.*, 1968).

Growth and sporulation. Unless stated otherwise, the cells were grown at 35 °C with vigorous aeration. Sporulation was induced by the replacement method of Sterlini & Mandelstam (1969). Cells growing in a rich medium containing hydrolysed casein and inorganic ions (CH medium) were transferred to sporulation medium containing L-glutamate and inorganic ions. Both media were supplemented with thymidine (20 µg ml⁻¹) and tryptophan (20 µg ml⁻¹). Culture growth was measured spectrophotometrically and bacterial dry weight was estimated from a calibration curve relating absorbance at 600 nm to bacterial dry weight.

Spore incidence. The number of phase-bright spores in 500 cells at 8 h after transfer to sporulation medium was counted in a phase-contrast microscope and values are expressed as a percentage.

Synchronization of chromosomes. The chromosomes were 'lined-up' by the method of Mandelstam & Higgs (1974). Cells were grown in CH medium (containing [³H]thymidine, 0.1 µCi ml⁻¹) at 35 °C to 0.1 mg dry wt ml⁻¹. The culture was then shifted to 45 °C for 45 min to allow replications in progress to complete but no new ones to start. Duplicate samples (1 ml) were taken at intervals and added to trichloroacetic acid (TCA; 1 ml; 10 %, w/v). The precipitates were collected on Whatman GF/C glass fibre filters, washed twice with TCA (5 %, w/v) containing unlabelled thymidine (300 µg ml⁻¹) and twice with ethanol. The filters were dried in an oven at 85 °C, transferred to scintillation vials containing 2-(4'-*tert*-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole (5 ml; 0.5 %, w/v, in toluene) and counted in a scintillation counter. These measurements showed that DNA synthesis had usually ended after 30 min.

Serine protease. The enzyme activity was measured as described by Dancer & Mandelstam (1975) with Remazol brilliant blue hide powder (Calbiochem) as substrate.

RESULTS

Potential for sporulation and for production of serine protease during synchronized chromosome replication. The chromosomes of *B. subtilis* ts-134 growing in hydrolysed casein medium were lined-up by shaking the culture at the restrictive temperature (45 °C) for 45 min (see Methods). The culture was then diluted with an equal volume of the same medium at 25 °C. This simultaneously halved the density of the culture and brought its temperature to 35 °C so that new replications could start. After 15 min the culture was returned to the 45 °C bath. This procedure allowed the cells to complete those replications that had started but not to begin any new ones.

Table 1. Incidence of sporulation in cells induced at different points in the chromosome replication cycle

The chromosomes of a temperature-sensitive *dna* mutant growing in rich medium were 'lined-up' and a synchronized replication was started by lowering the temperature (see text). At 15 min intervals, portions of the culture were centrifuged and the cells were transferred to a poor medium to induce sporulation. Spore incidence was measured after 8 h. Results from three experiments and mean values are given.

Replication time (min)	Spore incidence (%)
0	4, 8, 4 (5)
15	18, 20, 16 (18)
30	6, 9, 9 (8)
45	5, 6, 4 (5)

Fig. 1. Production of serine protease by cells induced to sporulate at different points in the chromosome replication cycle. Portions of a culture undergoing synchronized chromosome replication were transferred to a poor medium at 15 min intervals to induce sporulation (see Table 1). The resuspension volumes were adjusted so that all the subcultures had the same cell density. (This was necessary to compensate for the growth that occurred in the master culture after the zero time portion was removed). The four subcultures were sampled hourly for measurement of serine protease. Time of chromosome replication: 0 min, ○; 15 min, ●; 30 min, ▲; 45 min, □.

If synchronous replication had been initiated in the whole cell population during the 15 min period at 35 °C the DNA content of the cells should have doubled. Instead, the increase in a number of experiments varied from 60 to 90 %, presumably because a proportion of the cells failed to initiate replication. In the cultures transferred at 0 min this proportion was much higher and the increases in DNA were only one-third to one-half of those observed in the 15 min cultures. There was a correspondingly low degree of sporulation (see Table 1).

At intervals (0, 15, 30 and 45 min) during this synchronized replication, portions of the parent culture were centrifuged rapidly and the cells were transferred to sporulation medium (Sterlini & Mandelstam, 1969) supplemented with thymidine (20 $\mu\text{g ml}^{-1}$). The cultures were shaken at 35 °C and samples were taken at hourly intervals for measurement of absorbance, serine protease and, at 8 h, of spore incidence.

The incidence of spores, in keeping with the earlier observations of Mandelstam & Higgs (1974), was maximal in cells transferred at 15 min (Table 1). The incidence in the 30 min sample had fallen by over half and by 45 min the sporulation potential had decreased even further. By contrast, the serine protease measurement showed no such relationship (Fig. 1). Although there were differences in the length of the lag period before enzyme production started, the slopes of the curves during active synthesis were very similar. The differences observed were well within the day-to-day variations encountered in this type of experiment.

DISCUSSION

When *B. subtilis* cells are transferred from a good growth medium to a poor one a number of genetic loci, presumed to be operons and estimated at between 42 and 60, are successively activated (Hranueli *et al.*, 1974; Mandelstam, 1976; Piggot & Coote, 1976). Their expression culminates in the formation of the mature spore. The first morphological change clearly indicating that a cell has been switched into the sporulation pathway is the formation of an asymmetric septum (stage II) about 1.5 h after transfer of the cell to the step-down condition. It is preceded by the expression of about 10 operons (designated stage 0–I) which are apparently activated in a linear temporal sequence (see Piggot & Coote, 1976; Young &

Mandelstam, 1980). One of the first operons among these 10 is presumed to control production of protease.

Earlier work, referred to in the Introduction, showed that the cells are optimally receptive to the induction of sporulation about 15 min after the beginning of chromosome replication (see also Table 1). It was tacitly assumed that at this critical time point in replication the first operon in the sporulation sequence was turned on and that this led, in turn, to the sequential induction of the rest. The results that are described here show that this was a false assumption. Instead, it is apparent that cells exposed to step-down conditions at any stage in the replication cycle are equally able to synthesize protease. In addition, preliminary experiments with RNAase, another very early sporulation event, seem to show that synthesis of this enzyme is also independent of chromosome replication (S. Clarke & J. Mandelstam, unpublished).

Because these early events are turned on equally well in all the cells and not only in those that are going to sporulate it follows that the 'watershed' operon, i.e. the operon whose activation diverts the cells into the sporulation pathway, must be one of the stage 0-I operons concerned with a somewhat later event than the expression of protease.

It is important for our understanding of the induction of spore formation that this key operon should be identified. In practice, this requires that the induction of stage 0-I events later than protease should be studied in relation to the chromosome replication state of the cells. At present, unfortunately, no biochemical properties as easily measurable as this enzyme or RNAase are known to characterize the remainder of stage 0-I.

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