Pattern of RNA Transcription during *Bacillus subtilis* Spore Outgrowth

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During early outgrowth of *Bacillus subtilis* spores, there is a period of RNA and protein synthesis in the absence of DNA replication. Two mutants of *B. subtilis*, PB2442 (*gsp-4*) and PB2452 (*gsp-81*), were used to study the pattern of RNA synthesis during this period. The two mutants are temperature-sensitive in the outgrowth phase, and show a limited amount of incorporation of [³H]uridine at 47 °C. The RNAs synthesized during a 2 min pulse with [³H]uridine were hybridized to *Eco*RI-digested DNA, after agarose gel electrophoresis and transfer to nitrocellulose paper (Southern technique). For both mutants the transcripts synthesized at 35 °C at different times were different. Differences were also observed in the transcripts made at 47 °C. For both mutants, in the presence of chloramphenicol, the same hybridization pattern was obtained for RNAs pulse-labelled at different periods during outgrowth.

INTRODUCTION

The process of bacterial endospore germination and outgrowth is an interesting system for the study of the regulation of gene expression at a level of complexity intermediate between the bacterial operon and the eukaryotic cell. During outgrowth the synthesis of macromolecules starts with the synthesis of RNA, followed by the synthesis of proteins and, much later, by the synthesis of DNA (Balassa, 1965; Kobayashi *et al.*, 1965). Thus, in this system there is a period during which RNA and protein synthesis occur without concomitant DNA synthesis.

A strict programme of protein synthesis takes place during outgrowth, with different enzymic activities first appearing at different times (Steinberg & Halvorson, 1968). The order of expression of the genes is independent of their chromosomal location, as shown by the analysis of temperature-sensitive mutants (Albertini *et al.*, 1979) and by the use of chromosomal translocations (Yeh & Steinberg, 1978).

The synthesis of proteins is dependent on previous RNA synthesis, suggesting that different messenger RNAs should be present at different periods during outgrowth, if regulation occurs at the level of transcription. Hansen *et al.* (1970) have analysed the differences and similarities between mRNAs synthesized at different times during outgrowth of *Bacillus cereus* spores, by means of DNA hybridization experiments with labelled RNA and competition with unlabelled RNA. The data show that there is a fluctuation in the appearance of predominant unstable RNA species. More recently, Silberstein & Cohen (1978), using hybridization of pulse-labelled RNA to *Eco*RI DNA fragments in the presence of competing unlabelled RNA, found very few differences in the hybridization pattern of RNA pulse-labelled at different periods during outgrowth of *B. cereus* spores. In *Bacillus subtilis*, experiments involving hybridization of pulse-labelled RNA to separate H and L strands of DNA, coupled to competition with unlabelled RNA, have revealed that during the germination process unique RNAs are transcribed which are absent from exponential-phase cells, and changes in the transcription pattern occur during outgrowth (Margulies *et al.*, 1978; Setoguchi *et al.*, 1978).

The isolation of mutants defective only in the outgrowth phase of spore germination may help in elucidating the control mechanisms regulating this process (Albertini *et al.*, 1979). In the work described in this paper, we employed two mutant strains (bearing the mutations *gsp-4* and *gsp-81*) impaired in RNA synthesis during outgrowth at 47 °C to study the pattern of transcription during the outgrowth of spores of *B. subtilis*. The RNA, pulse-labelled at different times, was hybridized to electrophoretically separated *EcoRI* fragments of *B. subtilis* DNA (Southern, 1975). For both mutants we found that the transcripts synthesized at 35 °C at different times were different. Differences were also observed at 47 °C. For both mutants, in the presence of chloramphenicol, only an early portion of the programme was realized.

METHODS

Bacteria and phage. Two *Bacillus subtilis* strains that are temperature-sensitive in spore germination were used: PB2442 (*hisH2 trpC2 gsp-4*) (Albertini *et al.*, 1979) and PB2452 (*pyrA26 trpC2 gsp-81*). Strain PB2452 was constructed by marker congression in transformation, using DNA extracted from strain PB2427 (Albertini & Galizzi, 1975) and competent cells of the strain PB1715 (*pyrA26 lys-3 trpC2*): selection was for Lys⁺ and the transformants were scored for the thermosensitive *gsp* unselected marker. Strain PB1424 (*hisH2 trpC2 metD4*) was used to prepare unlabelled ribosomal RNA and DNA for the hybridization competition experiments. Labelled phage SPP1 DNA was prepared by phenol extraction from CsCl-purified phage previously labelled with [*methyl*-³H]thymidine (5 µCi ml⁻¹) by growing the phage on the bacterial strain PB566/1 (*thyA thyB*).

Media. For RNA labelling, M9 minimal salt medium (Clowes & Hayes, 1968) was diluted threefold with distilled water, supplemented with 0.2% (w/v) Casamino acids (Difco), 0.5% (w/v) glucose and 50 µg ml⁻¹ of each of the amino acids required for growth. For growth of strain PB1424 for the purification of DNA and ribosomal RNA, Penassay broth (antibiotic medium no. 3, Difco) was used. For the preparation of phage lysates, PS broth was used (Riva *et al.*, 1968).

Radioactive chemicals. [³H]Uridine (specific activity 25–30 Ci mmol⁻¹, 0.9–1.1 TBq mmol⁻¹) and [*methyl*-³H]thymidine (specific activity 18 Ci mmol⁻¹, 670 GBq mmol⁻¹) were obtained from Amersham.

Labelling of RNA. Spores, purified according to Siccardi *et al.* (1975) and heat-activated at 70 °C for 15 min, were resuspended in 200 ml of M9 medium at an A₅₆₀ of about 0.60 and incubated at the desired temperature. RNA was labelled at different times after the onset of spore germination by a 2 min pulse of [³H]uridine at a final concentration of 5 µCi ml⁻¹. Labelling was stopped by transferring the cultures to a salt/ice mixture and adding 50 ml of cold M9 medium and 50 µg sodium azide ml⁻¹. Cells were collected with centrifugation, washed twice with cold M9 medium and stored at –20 °C.

Purification of RNA. Labelled bacteria were resuspended in 2 ml of DNAase buffer containing 10 mM-sodium acetate, 50 mM-NaCl, 1 mM-MnCl₂ and 50 µg pancreatic deoxyribonuclease ml⁻¹ (ribonuclease free, Boehringer). Glass beads (0.1 mm diam., 2 g) were added and the suspension was sonicated in pulses for a total of 15 min in a salt/ice bath. After sonication the broken cells were incubated at 0 °C for 90 min. The DNAase reaction was stopped by adding EDTA at a final concentration of 2 mM. RNA was extracted at 60 °C with 2 ml of water-saturated phenol. The aqueous phase, separated by centrifugation, was re-extracted twice and, after addition of KCl to 0.5 M final concentration, was filtered (Selectron filter BA85, 0.45 µm pore size; Schleicher & Schüll). The RNA was precipitated with 3 vol. cold ethanol. After standing at least one night at –20 °C the precipitate was collected by centrifugation at 15 000 rev. min⁻¹ for 15 min and dissolved with 1 ml of 6× SSC buffer (0.9 M-NaCl, 0.09 M-trisodium citrate).

The unlabelled ribosomal RNA was prepared from ribosomes according to Traub *et al.* (1971).

EcoRI digestion. DNA was extracted from cells as described by Saito & Miura (1963) and treated with sufficient *EcoRI* restriction endonuclease (Bethesda Research Laboratories and Miles Laboratories) to give complete digestion in 3 h at 37 °C. The reaction mixture was prepared according to the manufacturer's instructions. Restriction fragments were subjected to electrophoresis in 1% (w/v) agarose (Bio-Rad) slab gels with tris/acetate buffer, as described by Cohen *et al.* (1973), and in the presence of 1 µg ethidium bromide ml⁻¹. Electrophoretic patterns were visualized with ultraviolet light.

RNA–DNA hybridization on nitrocellulose strips. The *EcoRI*-digested *B. subtilis* DNA fragments were denatured on the gel and transferred to a nitrocellulose membrane (Selectron BA85; Schleicher & Schüll) as described by Southern (1975). Transfer efficiency was checked using ³H-labelled SPP1 DNA and counting the radioactivity bound to nitrocellulose. The efficiency varied between 50 and 100%. Strips (0.5–1 cm wide) containing about 5 µg of *EcoRI*-digested *B. subtilis* DNA were cut and baked in a vacuum oven at 80 °C for 2 h. For hybridization experiments, strips were incubated at 66 °C for 18 h in 1.5 ml of 6× SSC with 0.5 × 10⁵–2 × 10⁵ c.p.m. (5–30 µg) of [³H]uridine-labelled RNA in the presence of 200 µg of unlabelled ribosomal RNA.

Subsequently, the strips were washed with 2 ml of $6 \times \text{SSC}$ at 66°C for 1 h, treated with 2 ml of $6 \times \text{SSC}$ containing $25 \mu\text{g}$ pancreatic ribonuclease A ml^{-1} and $10 \text{ U T1 ribonuclease ml}^{-1}$ (Boehringer) for 1 h at 20°C , then washed with 2 ml of $6 \times \text{SSC}$ for 2 h at 20°C and dried for 2 h at 80°C .

Fluorography. Strips were dipped in a solution of 20% (w/v) 2,5-diphenyloxazole in toluene, dried in the air and subjected to fluorography as described by Laskey & Mills (1975) using Kodak-X-Omat R film (XR-5) pre-exposed to a single flash of light filtered through a Deep orange, Wratten no. 22 filter (Kodak). The fluorography was performed at -70°C for 15–60 d. ^3H -labelled SPP1 DNA *EcoRI* fragments subjected to agarose electrophoresis, Southern transfer and fluorography were used to estimate molecular weights.

RESULTS

Hybridization of pulse-labelled RNA during outgrowth at 35°C

Total chromosomal DNA of *B. subtilis* was digested with *EcoRI* endonuclease and the fragments were separated by agarose gel electrophoresis. The DNA was denaturated and transferred to nitrocellulose paper by the method of Southern (1975). RNA pulse-labelled with $[^3\text{H}]$ uridine was hybridized to the DNA paper strips in the presence of unlabelled ribosomal RNA. In our previous work (Galizzi *et al.*, 1976) we have shown that during outgrowth the RNA labelled in a 2 min pulse is 50% ribosomal. Excess unlabelled ribosomal RNA was therefore added in order to reduce hybridization of ^3H -labelled ribosomal RNA. As shown by Silberstein & Cohen (1978), ribosomal RNA gives 18 hybridization bands to *EcoRI*-digested DNA in *B. cereus* and we have obtained a similar result in *B. subtilis* (data not shown). Following hybridization, the nitrocellulose strips were saturated with 2,5-diphenyloxazole and fluorography was performed. Hybridization with RNA of the outgrowing spores of mutant PB2442 (*gsp-4*) incubated at 35°C and pulse-labelled at 13, 25 and 62 min gave the fluorograms shown in Fig. 1A. Several reproducible hybridization bands could be seen and differences were observed between the samples pulse-labelled at 25 and 62 min from the onset of germination. A similar evolution in the pattern of transcription was seen with the spores of mutant PB2452 (*gsp-81*), incubated at 35°C and pulse-labelled at 13, 25 and 45 min (Fig. 1B).

*Transcription during outgrowth of mutant PB2442 (*gsp-4*) spores at 47°C*

Since the spores of mutant PB2442 (*gsp-4*) are impaired in RNA synthesis during outgrowth at 47°C , we have studied the transcripts present at different time intervals after the onset of germination at the non-permissive temperature. Only one difference was observed between the fluorograms corresponding to RNA pulse-labelled at 13, 25 and 62 min: one band of about 4.6 megadaltons was absent at 13 min, synthesized at 25 min and had disappeared at 62 min (the band is indicated by ◀ in Fig. 3). Thus, at the non-permissive temperature only a variation in the pattern of transcription takes place in mutant PB2442 (*gsp-4*).

To obtain a further parameter that could be useful in providing a picture of transcription during spore outgrowth, the RNAs were also pulse-labelled in the presence of an inhibitor of protein synthesis. In the presence of chloramphenicol at 47°C (47°C -CM-RNA) the same pattern was obtained at different times during outgrowth. Hybridization patterns of the 47°C -CM-RNAs are shown in Fig. 2 together with the pattern obtained with spores incubated without chloramphenicol at 47°C (47°C -RNA) and labelled at 13 min; no differences were observed. This suggests that in the absence of protein synthesis no transition can occur and the outgrowing spores perform only the first phase of the transcription programme.

The hybridization bands obtained in the three different conditions (35°C , 47°C , $47^\circ\text{C} + \text{CM}$) at the same labelling time (25 min) are shown together in Fig. 3. There were differences between the bands for 35°C and the other two conditions. One band present at 47°C was missing at $47^\circ\text{C} + \text{CM}$ and one band present at $47^\circ\text{C} + \text{CM}$ was missing at 47°C , again

Fig. 1

Fig. 1. Hybridization of RNA pulse-labelled with [^3H]uridine during spore outgrowth at 35 °C. DNA was digested with *Eco*RI endonuclease and the fragments were separated by agarose gel electrophoresis. After transfer to nitrocellulose paper the RNA pulse-labelled for 2 min was hybridized in the presence of 200 μg of unlabelled ribosomal RNA, as described in Methods. The hybridization pattern was visualized by fluorography.

A: RNA from mutant PB2442 (*gsp-4*) labelled at 11–13 min (a), 23–25 min (b) and 60–62 min (c) after inoculation. B: RNA from mutant PB2452 (*gsp-81*) labelled at 11–13 min (d), 23–25 min (e) and 43–45 min (f) after inoculation. The positions of molecular size markers (in megadaltons) of phage SPP1 [^3H]DNA digested with *Eco*RI endonuclease are shown. The $\triangleleft$ indicate differences in the banding patterns observed at different labelling times.

Fig. 2

Fig. 2. Hybridization to *Eco*RI DNA fragments of RNA pulse-labelled during outgrowth at 47 °C of spores of mutant PB2442 (*gsp-4*) in the presence and in the absence of chloramphenicol. RNA labelled at 11–13 min in the absence of chloramphenicol (a); RNA labelled at 11–13 min (b), 23–25 min (c) and 60–62 min (d) in the presence of chloramphenicol (50 $\mu\text{g ml}^{-1}$) from the time of inoculation.

indicating that at 47 °C the mutant is blocked very early and transcription can go only one step further than the transcription performed in the absence of protein synthesis.

Transcription during outgrowth of mutant PB2452 (gsp-81) spores at 47 °C

Spores of the mutant PB2452 (*gsp-81*) are also altered in RNA synthesis during outgrowth at 47 °C. From temperature-shift experiments, different temperature-sensitive periods have been assigned to the two mutants, the *gsp-4* mutation affecting outgrowth earlier than *gsp-81* (Albertini *et al.*, 1979).

The hybridization patterns of RNA pulse-labelled at 13, 25 and 45 min of outgrowth at 35 °C are shown in Fig. 1B. Differences were seen among the transcripts present in the different samples. A changing pattern was also seen at the non-permissive temperature (Fig. 4). Two differences were seen between the samples pulse-labelled at 13 min and 25 min and one further difference between those pulse-labelled at 25 min and 45 min. Thus, in this mutant, even at the non-permissive temperature, there was an evolution in the pattern of RNA synthesis though the changes were fewer than those observed at 35 °C. In the presence of chloramphenicol the same patterns of hybridization bands were seen with RNA pulse-labelled at 13, 25 and 45 min. The hybridization patterns obtained with RNA

Fig. 3

Fig. 4

Fig. 5

Fig. 3. Hybridization to *EcoRI* DNA fragments of RNA pulse-labelled at 23–25 min during outgrowth of spores of mutant PB2442 (*gsp-4*). Spores incubated at 35 °C (a), at 47 °C (b) and at 47 °C in the presence of chloramphenicol (50 µg ml⁻¹) from the time of inoculation (c). The ◁ indicate differences in the banding patterns between (a) and (b); ◼ indicates the difference between (b) and (c).

Fig. 4. Hybridization to *EcoRI* DNA fragments of RNA pulse-labelled during outgrowth at 47 °C of spores of mutant PB2452 (*gsp-81*). RNA labelled at 11–13 min (a), 23–25 min (b) and 43–45 min (c) after inoculation. The ◁ indicate differences in the banding patterns between (a) and (b); ◼ indicates the difference between (b) and (c).

Fig. 5. Hybridization to *EcoRI* DNA fragments of RNA pulse-labelled at 23–25 min during outgrowth of spores of mutant PB2452 (*gsp-81*). Spores incubated at 35 °C (a), at 47 °C (b) and at 47 °C in the presence of chloramphenicol (50 µg ml⁻¹) from the time of inoculation (c). The ◁ indicate differences in the banding patterns between (a) and (b); ◼ indicate the differences between (b) and (c).

pulse-labelled at 25 min during outgrowth at 35 °C, 47 °C and 47 °C + CM are shown in Fig. 5. Differences were seen between the 35 °C-RNA and the 47 °C-RNA and between the 47 °C-RNA and the 47 °C-CM-RNA. In the last case at least three bands present at 47 °C were missing from the sample labelled in the presence of chloramphenicol. Quantitative differences were also seen in the molecular mass range of 0.88 to 1.77 megadaltons, in particular between 47 °C-RNA and 47 °C-CM-RNA. Thus, at 47 °C in absence of protein synthesis, the RNA species transcribed are a subset of the transcripts synthesized at the non-permissive temperature.

DISCUSSION

Even in the earliest period (13–15 min) considered, we did not observe the exclusive synthesis of stable RNA. Similar results were obtained by Margulies *et al.* (1978). For both strains at 35 °C a programme of changes in the pattern of RNA hybridization bands takes place during outgrowth. This is consistent with the data of Hansen *et al.* (1970) and the more recent work of Margulies *et al.* (1978) and Setoguchi *et al.* (1978). At the non-permissive temperature the spores of the strain bearing the *gsp-4* mutation can perform a very limited amount of change in transcription; in fact only one difference could be detected, namely, a

Fig. 6. Interpretation of the results. In both mutants, during outgrowth at 47 °C in absence of protein synthesis only a limited number of DNA regions can be transcribed: there appears to be no difference between the two mutants in this respect. At 47 °C, mutant PB2442 (*gsp-4*) is affected in outgrowth at an early stage, and only a second step of the transcription programme can be carried out. Mutant PB2452 (*gsp-81*) is blocked later during outgrowth and is capable of performing one further step of the programme.

band absent at 13 min was synthesized at 25 min and disappeared at 62 min. The pattern observed at 47 °C is almost identical to that seen with RNA pulse-labelled at 35 °C in the presence of chloramphenicol. In the latter case no evolution could be detected during the time span considered. Thus, the *gsp-4* mutation affects outgrowth transcription at an early stage of the programme. The block is one step further removed with respect to the block in transcription obtained in the presence of chloramphenicol (Fig. 3). We would expect that during outgrowth at the non-permissive temperature, few proteins are synthesized by the spores with the *gsp-4* mutation. Preliminary analyses of the newly synthesized proteins by two-dimensional electrophoresis are completely consistent with this prediction (data not shown).

The second mutation examined (*gsp-81*) has a less severe effect on the transcription of spores outgrowing at 47 °C. Despite the fact that RNA synthesis is impaired, there is still an evolution in the pattern of hybridization bands, and the mutant spore appears to be capable of performing a number of switches in the programme of RNA synthesis. In this mutant we have previously shown that in the presence of chloramphenicol more RNA is accumulated at 47 °C and the rate of synthesis is maintained at a higher level for a longer period compared with synthesis at 47 °C in the absence of the drug (Albertini & Galizzi, 1975). By quantitative hybridization and competition we showed that only some of the species pulse-labelled were common to 47 °C-RNA and to 47 °C-CM-RNA (Galizzi *et al.*, 1976). From the present data (Fig. 5) it appears that the hybridization pattern of 47 °C-RNA is more complex than that of 47 °C-CM-RNA, and that all differences are in the presence of more bands rather than in the absence of any bands.

It has been calculated that the *EcoRI* endonuclease cuts *B. subtilis* DNA into about 500 fragments (Dean & Halvorsen, 1976), whilst on agarose gels only about 100 bands can be resolved. This means that, on average, each band must comprise several DNA fragments. Consequently, the differences observed between hybridization patterns represent a minimal estimate of the differences between the RNAs tested. On the other hand, similar patterns of hybridization do not necessarily mean that the RNAs under scrutiny are the same; in this case one would expect to obtain different intensities in the hybridization bands. We have purposely avoided considering such differences, due to difficulties involved in a quantitative comparison of samples necessarily hybridized separately and in normalizing the intensities of the different bands. Thus, we have examined only the quality (i.e. electrophoretic mobility) of the hybridization bands obtained by fluorography.

The overall picture emerging from the results presented in this paper is shown schematically in Fig. 6. In the presence of chloramphenicol only a limited number of

transcripts are present. The realization of the first stage of the programme does not require protein synthesis and the hybridization patterns for both mutant strains are identical (data not shown). The strain with the *gsp-4* mutation is blocked very early during outgrowth and can perform only one further step in the transcription programme. The *gsp-81* mutation blocks transcription at a later stage during outgrowth, allowing the realization of a larger part of the programme.

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