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Ribonucleic Acid Polymerase in a Thermosensitive Sporulation Mutant (ts-4) of *Bacillus subtilis*

C. ORREGO,[1] P. KERJAN, M. C. MANCA DE NADRA, AND J. SZULMAJSTER

Laboratoire d'Enzymologie du Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France

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Partially synchronized cultures of a *Bacillus subtilis* thermosensitive sporulation mutant (ts-4) and the 168 trp^- thy^- (168tt) parental strain were infected with the virulent phage φe at various times during their growth cycle at 30 and 42 C (permissive and restrictive temperatures, respectively). It was shown that at the restrictive temperature the burst size in the parental strain was two- to threefold lower than in the ts-4 mutant. No such difference was observed at the permissive temperature. However, the time at which this difference was observed excludes a correlation between the burst size and initiation of the sporulation process. It was further found that the capacity to transcribe in vitro phage φe deoxyribonucleic acid by partially purified ribonucleic acid (RNA) polymerase from both strains decreased sharply if the source of enzyme was sporulating cells instead of vegetative ones. However, a similar decrease, although to a lesser extent, was observed with the RNA polymerase isolated from stationary-phase cells of the ts-4 mutant grown at the nonpermissive temperature, or with the enzyme derived from several other zero-stage sporulation mutants. At no time was a structural modification in the β subunits of the RNA polymerase observed during growth of the sporulating bacteria. We have also shown that, in addition to the relatively low specific activity of the RNA polymerase, the level of the intracellular protease activity is about 15-fold lower in the ts-4 mutant grown at the restrictive temperature than that of the parental strain grown at the same temperature. At the permissive temperature no such difference was observed between these two strains. However, the present data do not allow us to establish a correlation among the low content of intracellular protease, the weak specific activity of the RNA polymerase, and the loss of the sporulation capacity in the ts-4 mutant grown at the restrictive temperature.

Previous studies have shown that the ts-4 thermosensitive sporulation mutant of *Bacillus subtilis* is able to grow equally well at 30 and 42 C but is unable to sporulate at the higher temperature (23). Electron microscopy has shown that this mutant is blocked at stage 0 of spore development. Further studies based on shifting cultures of the ts-4 mutant from 30 to 42 C and from 42 to 30 C at different stages of the growth cycle have also shown that the event affected at the high temperature takes place at a very early stage of spore development. As a consequence of this early block in the sporulation process, the subsequent developmental program in the mutant is arrested. A pleiotropic phenotype is therefore produced, in which the ability to catalyze the late biochemical reactions required for spore formation is lost. This loss includes the enzymes involved in the biosynthesis of dipicolinic acid, essential for sporulation.

The main question, however, to be answered concerns the exact nature of the alteration of the initial sporulation functions in this mutant grown at the restrictive temperature. The present study was designed to examine the possibility of an alteration in the ribonucleic acid (RNA) polymerase function in the ts-4 mutant when growth occurred at the restrictive temperature. In the course of these studies, no structural modification of the β subunit of the

[1] Present address: Departmento de Quimica, Universidad Catolica de Valparaiso, Casilla 4059, Valparaiso, Chile.

polymerase was observed during sporulation either in the mutant strain grown at the permissive temperature or in the parental strain at any temperature.

MATERIALS AND METHODS

Organisms. The following organisms were used: *B. subtilis* 168, *trp*⁻, *thy*⁻ (168tt); ts-4, a thermosensitive sporulation mutant derived from 168tt (23); 12A (20); BS-21, *arg*⁻, Rif^r, Spo⁻ (from N. Strauss); BS-22, *trp*⁻, Rif^r, Spo⁻; *B. subtilis* 3610, wild type (ATCC 6051); and phage ϕe. The last two organisms were received from R. Losick.

Media. Nutrient broth (Difco) was used for precultures and production of *B. subtilis* 3610 spores. In some cases, Pennassay broth (Difco) was also used for precultures.

Double-strength nutrient broth (Difco) containing 0.1% glucose was used for growth and sporulation cultures (8). When necessary, tryptophan and thymine (50 μg/ml) were added to the medium. The double-strength medium was sterilized in the absence of the inorganic salts. The mixture of sterile salts was added before use. This procedure prevented agglutination of cells during the stationary phase. Difco nutrient agar was used for bacterial plating.

LB broth, LB agar, and top agar were used to assay the phage. Phage plaques were revealed by incubation at 30 C for 17 h.

Phage stocks were maintained and, when necessary, diluted in ϕe buffer containing, per liter: 5 g of NaCl and 2.4 g of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4 (sterilized together) plus 0.25 g of $MgSO_4$ $7H_2O$ and 0.10 g of gelatin added separately.

Growth and sporulation were carried out in Fernbach flasks containing a volume of medium not exceeding one-tenth the volume of the flask, and cultures were incubated at the desired temperature with vigorous rotary shaking (250 to 300 rpm). Growth was followed with a Zeiss spectrophotometer by measuring the absorbancy at 650 nm. Spores or refractile bodies were counted either by plating the cells after heating (10 min at 80 C) or directly in a Petroff-Hausser counting chamber under a phase-contrast microscope. Sporulation levels were determined by the ratio of the number of heat-resistant spores to the number of vegetative cells (s/c) present at the beginning of the stationary phase (maximal absorbancy). Both types of cells were counted by plating on nutrient agar.

Spore suspensions used as an indicator for phage plating were washed four times in distilled water and kept at 4 C until use.

Production of phage ϕe stocks. High-titer phage stocks were prepared as follows. An overnight culture of *B. subtilis* 3610 in LB broth was diluted 1:20 with fresh broth. Growth was followed with 400 ml of this medium in a Fernbach flask subjected to rotary shaking at 37 C until the optical density (OD) reached 1.3 (5×10^8 cells/ml), at which point phage ϕe was added to approximately 2.5×10^7 plaque-forming units (PFU) per ml (the initial ratio of phage to bacteria should not surpass 0.1). The culture was shaken until lysis was complete (2 to 3 h). After storage overnight at 4 C, the lysate was centrifuged for 5 min at $12,000 \times g$ in a Sorvall centrifuge, followed by the centrifugation of the supernatant fluid for 2 h at $12,000 \times g$. The pellet was covered with 5 ml of ϕe buffer and suspended by gently whirling from time to time. After 24 to 48 h, the suspension was pooled and centrifuged for 5 min at $9,750 \times g$; then the supernatant fluid was diluted with ϕe buffer to half its original volume and centrifuged for 2 h at $12,000 \times g$ in a Sorvall centrifuge. The pellet was suspended in a small volume and centrifuged for 5 min at $12,000 \times g$; the supernatant fluid was stored over a crystal of thymol. A total of about 10^{13} PFU was usually recovered from 1.6 liters of original lysate.

Preparation of ϕe DNA. The phage suspension was adjusted to a concentration of 10^{12} PFU/ml with ϕe buffer, in a glass-stoppered bottle, and 0.1 volume of 1 M Tris (pH 9.0)-5% sodium dodecyl sulfate (SDS; recrystallized) was added. The mixture was whirled by hand for 15 min at room temperature. Then sodium *p*-aminosalycilate was added to a final concentration of 50 μg/ml followed by the addition of 1 volume of a prewarmed phenol mixture (10 parts of freshly distilled phenol, 1.1 parts of water, 1.4 parts of freshly distilled *m*-cresol, and 0.1% 8-hydroxyquinoline. Deproteinization was carried out by gently shaking for 30 min at 30 C, and the deoxyribonucleic acid (DNA) solution was separated by low-speed centrifugation for 5 min at room temperature. The DNA was dialyzed extensively against 0.1 M Tris-hydrochloride (pH 7.9)-0.5 mM ethylenediaminetetraacetate (EDTA) and kept in the cold after addition of a drop of chloroform. The DNA concentration was measured by OD at 260 nm. From 5×10^{13} PFU, 13.3 mg of DNA ($OD_{280/260} = 0.51$) was obtained.

B. subtilis DNA was prepared according to Marmur (13) with an additional treatment by T_1 ribonuclease. Denatured DNA was prepared by heating for 10 min at 100 C in 0.15 M NaCl-0.015 M sodium citrate, pH 7.0, followed by rapid cooling.

Phage assay and plating conditions. A sample of an appropriate dilution (ratio of phage to bacteria should be 0.1 or less) of the phage suspension was added to 2 ml of an LB culture of *B. subtilis* 3610 at 37 C (10^8 colony-forming units/ml), and adsorption was allowed for 10 min with shaking. For colony counts, bacteria and phage were plated on LB agar. For plaque-forming assay, phage or infected bacteria were mixed with 2×10^8 spores of *B. subtilis* 3610 in 2.5 ml of top agar.

Single-step growth. Cells were infected at a phage to bacteria ratio of 0.1 or less, and adsorption was allowed for 10 min at the temperature of the experiment with reciprocal shaking. The adsorption mixture was diluted into phage antiserum (1:10 dilution) of an activity such as to inactivate 99.9% of the phage within 5 min at the temperature of the experiment. After dilution of at least 100-fold, a 0.10-ml sample was assayed for infective centers. The dilutions were shaken and removed at intervals to determine the average burst size.

Electrophoresis in SDS-polyacrylamide gels.

Analysis of RNA polymerase extracts was performed on gels (10 cm long) of 5% (wt/vol) acrylamide according to the method of Shapiro, Viñuela, and Maizel (18). So that protease degradation during electrophoresis in presence of SDS could be prevented, the following mixture was made and heated for 5 min at 75 C: 20 to 30 μliters of the protein sample, 5 μliters of 0.1 M diisopropylfluorophosphate (DFP; in isopropanol), 10 μliters of 0.1 M EDTA, 100 μliters of 0.01 M sodium phosphate, pH 7.2, 10% glycerol, 1% β-mercaptoethanol, 0.1% SDS, and 5 μliters of 0.2% (vol/vol) bromophenol blue as indicator. The electrode compartments were filled with a buffer containing 0.1 M sodium phosphate, pH 7.2, and 0.1% SDS. Electrophoresis was carried out at room temperature at a constant current of 8 mA/gel for 22 h. This permitted about 1 mm of separation between the β subunits. The tracking dye and some protein material move into the lower buffer compartment. Gels were stained with a 0.12% (wt/vol) solution of Coomassie Blue in methanol-acetic acid-water (5:1:5, vol/vol) for 20 min. The gels were destained electrophoretically in 7.5% acetic acid.

Electrophoresis in normal gels. Electrophoresis in normal gels (nondissociating conditions) was carried out at 4 C in a buffer containing 0.005 M Tris-glycine, pH 7.9, 2 mM $MgCl_2$, and 1 mM dithiothreitol (DTT; 7).

For the separation of the intracellular from the extracellular protease activity, electrophoresis was carried out on bacterial crude extracts on 7% gels (9 cm long) with a constant current of 3 to 3.5 mA/gel until the tracking dye was at the bottom of the gel.

Partial purification of the RNA polymerase. The method used was designed to prevent proteolytic action during the isolation procedure.

Cells were washed twice with 0.01 M Tris buffer (pH 7.9) containing 0.02 M $MgCl_2$, 1 M KCl, 5 mM titriplex-Mg, 10% glycerol, and 0.01 M 2-mercaptoethanol (buffer A), and once with the same buffer containing 0.05 M KCl. Cells were frozen and kept at −20 C until use.

The cells were suspended in the following buffer (protease inhibitors prepared just before use): 0.01 M Tris (pH 7.9), 0.02 M $MgCl_2$, 5 mM titriplex-Mg, 5 mM β-mercaptoethanol, 5% glycerol, 3.5 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1 mM DFP, 0.1 M KCl, and 1 mg of macaloid/ml (buffer B). The suspension was subjected to Mini-Mill treatment in presence of glass beads (see footnote to Table 1). Cell breakage was followed by a 15-min centrifugation at 17,300 × *g* in an SS34 Sorvall rotor. Ribosomes were removed after centrifugation for 2 h at 100,000 × *g* in a Spinco centrifuge (rotor 40). Proteins in the supernatant fluid were precipitated by the addition of 25.6 g of ammonium sulfate per 100 ml. The pH was adjusted by addition of 0.05 ml of 1 M NaOH per 10 g of ammonium sulfate. The solution was stirred for 30 min, and the precipitate was removed by centrifugation at 12,000 × *g* for 10 min in an SS34 rotor. Then 14.4 g of ammonium sulfate per 100 ml was added to the supernatant fluid to give a 60% saturated solution. The precipitate was centrifuged for 45 min and the pellet was dissolved in 0.5 to 2 ml of the following buffer: 10 mM Tris (pH 7.9), 10 mM $MgCl_2$, 5 mM titriplex-Mg, 5 mM β-mercaptoethanol, 50 mM KCl, 3.5 mM PMSF, 0.2 mM DFP, and 50% glycerol (buffer C). All enzyme dilutions were done in this buffer.

RNA polymerase assay. If not otherwise indicated, the mixture for the RNA polymerase assay contained, in 0.25 ml: 0.04 M Tris-hydrochloride buffer (pH 7.9), 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 0.4 mM KPO_4, 0.15 M KCl, 0.5 mg of bovine serum albumin/ml, 0.18 mM each of guanosine, cytidine, uridine, and ^{3}H-adenosine triphosphates (20,000 to 40,000 counts per min per nmol), 4 μg of phage φe DNA or poly deoxy-(adenylate-thymidylate) [poly d(AT)], and 20 to 30 μg of enzyme. The enzyme activity in the ammonium sulfate fraction was linear up to about 160 μg of protein/ml.

The φe DNA-directed activity was measured with $MgCl_2$, and poly d(AT)-directed activity was measured with $MnCl_2$ (2).

The assay mixture was incubated for 10 min at 34 C, and the reaction was stopped with 1 ml of 10% trichloroacetic acid containing 0.20 ml of 0.1 M Na-pyrophosphate and chilled in ice. After 20 min, the precipitates were collected on GF/C Whatman filters, washed extensively with 2.5% trichloroacetic acid-0.01 M Na-pyrophosphate solution followed by 1 to 2 ml of distilled ethanol, and dried. The radioactivity was counted in a liquid scintillation counter.

One unit of enzyme activity incorporates 1 nmol of adenosine monophosphate under the conditions described above.

Intracellular protease activity from B. subtilis. Intracellular protease activity from *B. subtilis* was measured according to Millet (15). Cells were collected, extensively washed as for the preparation of cells for the RNA polymerase extraction, and kept at −20 C until use.

A 100-mg amount (packed wet weight) of cells was suspended in 2.5 ml of 0.2 M Tris (pH 7.3)-2 mM $CaCl_2$, and the cells were disrupted by treatment at −4 C for 3 min at about 65 W (Branson Sonifier). The extract was centrifuged for 15 min at 17,300 × *g* in an SS34 Sorvall head, and the supernatant fluid was kept at −20 C. The assay mixture contained, in 3 ml: 0.2 M Tris (pH 7.3), 2 mM $CaCl_2$, 15 mg of azocoll (Calbiochem), and 50 to 1,000 μg of protein. Several protein concentrations from each extract had to be tested to find the region of linear dependence on protein concentration.

The mixture was incubated at 37 C with constant agitation to achieve homogeneous dispersion of the azocoll. The mixture was then filtered through Whatman no. 1 filter paper, and the absorption was measured at 520 nm against a blank with no protein. One unit of activity corresponds to the hydrolysis of 1 μg of azocoll/min at 37 C. Complete hydrolysis of 5 mg of azocoll/ml gives an OD at 520 nm of 2.32.

Analysis of the β subunits of the RNA polymerase in B. subtilis crude lysates. The method used is based on a modification of that applied by Matzura, Molin, and Maaløe (14) for *Escherichia coli* lysates.

The pellet of cells (60 to 70 OD_{650} units) was resuspended in 1.0 ml of 0.01 M Tris buffer (pH 7.6)

containing 0.01 M $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 M KCl, 3.5 mM PMSF, 0.2 mM DFP, 5 mM titriplex-Mg, and 20% sucrose. To this suspension, 300 μg of lysozyme was added, and the mixture was incubated for 20 min at 37 C. This was followed by a low-speed centrifugation at 4 C to collect the protoplasts. The pellet was suspended in 0.9 ml of the following buffer: 0.005 M Tris-glycine (pH 7.9), 1 mM DTT, 2 mM $MgCl_2$, 3.5 mM PMSF, 5 mM titriplex-Mg, and 0.2 mM DFP.

The suspension was then disrupted by sonic oscillation for three 30-s periods with 1-min intervals between, and the cell debris was removed by centrifugation for 15 min at 17,300 $\times$ g in an SS34 rotor. The supernatant fluid was kept in 50% glycerol at -20 C. A sample of 0.20 ml was first subjected to normal gel electrophoresis (6% gel, 13 cm long) at 2.5 mA/gel until the tracking dye reached 2 to 3 mm from the bottom of the gel. This electrophoresis separated the DNA-RNA polymerase complex (which only slightly enters the gel) from the rest of the cellular material. The 2 mm on top of the gel containing the RNA polymerase activity were separated from the rest of the gel, homogenized in 0.2 ml in the Tris-glycine buffer, and again subjected to electrophoresis in SDS gel as previously described. Under these conditions, the β subunits were clearly visible and no contaminant materials were seen above the 110,000 dalton region. This technique can be improved by addition of a stacking gel (3 cm long, containing 3% [wt/vol] acrylamide and 0.25% [wt/vol] N,N-4-methylenebisacrylamide) in the SDS run.

Protein concentrations were estimated by the method of Lowry et al. (11), with crystalline bovine serum albumin as standard.

RESULTS

Synchronization with respect to sporulation. The experiments to be described were carried out on cultures partially synchronized with respect to sporulation (8). This was achieved by two transfers of cells that had just reached stationary phase on rich nutrient broth medium to fresh medium at a cell concentration that would not allow more than two or three cell divisions at exponential rate. The results of a typical experiment are illustrated in Fig. 1. In this experiment, synchronization of the sporulating culture was followed by the determination of the percentage of refractile bodies and a sporulation morphological stages, respectively, by phase-contrast microscopy and electron microscopy. It appears that, under these conditions of growth, at T_0 generally about 85 to 90% of the bacterial population enters stage 0–I (formation of axial filament from the two nuclei in the vegetative cell). As sporulation proceeded, there seemed to be a scattering of all developmental stages. However, at T_5-T_6 when the cells reached stage V-VI (appearance of refractility), a rapid increase in the frequency of the last stages was observed.

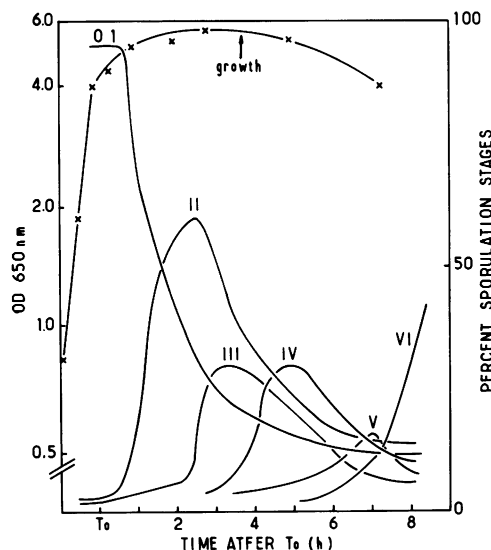

FIG. 1. *Distribution of morphological sporulation stages in a partially synchronized culture of Bacillus subtilis (see text). Starting from T_0 (end of exponential growth), samples were removed at hourly intervals for electron microscopy.* ($\times$) *Growth curve.*

Course of phage infection of the B. subtilis 168tt parental strain and of the ts-4 mutant. It was shown by Sonenshein and Roscoe (19) that, when vegetative cells of *B. subtilis* were infected by the virulent phage ϕe, they gave rise to a large progeny. However, when sporulating cells were infected by the same phage, cessation of the synthesis of at least three early phage products was observed, and consequently the bacteria no longer supported phage multiplication. Losick and Sonenshein (10) have further shown by in vitro experiments using partially purified RNA polymerase isolated from both vegetative and sporulating cells that the vegetative enzyme was able to transcribe either ϕe DNA or poly d(AT), whereas the RNA polymerase from sporulating cells was able to transcribe only poly d(AT) and not the phage DNA.

In investigating the nature of the defect in the ts-4 mutant grown at the restrictive temperature, it was important to determine whether a similar phenomenon takes place by following the course of infection of this mutant at 42 C. To compare the parameters of a single-step growth cycle of ϕe, we infected the 168tt strain at 30 and 42 C (Fig. 2a and b). It can be seen that at 30 C the burst was completed 150 min after infection, whereas at 42 C this was achieved at 110 min. At the latter temperature the burst was 700, compared with 590 at 30 C.

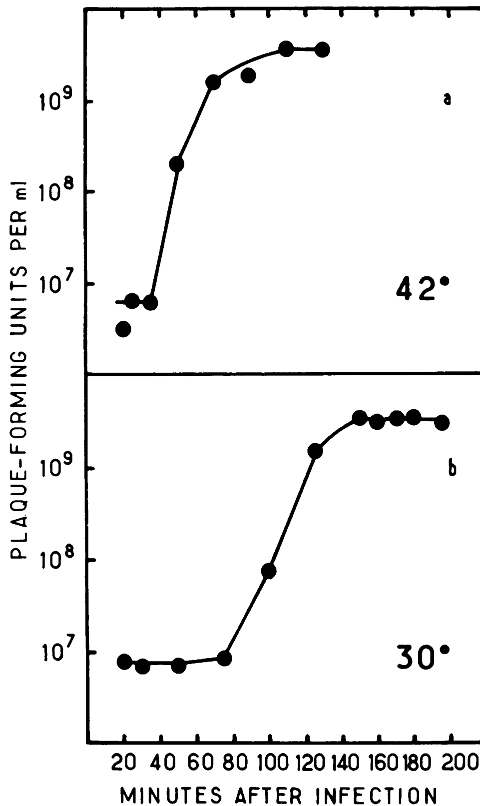

FIG. 2. *Phage φe single-step growth curves of Bacillus subtilis 168tt growth at 42 C (a) and 30 C (b) and infected at T_0.*

Figure 3a shows the average burst size of the parental strain and of the mutant as a function of time during the growth cycle at 42 C. At this temperature three patterns of infectivity can be observed. At the late logarithmic phase and during the stationary phase (T_7-T_{10}), a two- to threefold difference was observed in the average burst size between the mutant and the parental strain. This difference seemed to disappear at an intermediate time around T_2-T_5 and decreased rapidly at later times around T_{14}. At 30 C (Fig. 3b) no difference in the pattern of infection was observed between these two strains during growth and sporulation. It is important to note that in this experiment at 30 C the doubling time and the time of appearance of refractile bodies were about three times lower than at 42 C. Thus, at the latter temperature in this experiment, 50% of refractile bodies were seen at T_7, whereas at 30 C the same percentage was obtained at about T_{22}. For this reason, sampling at 30 C in the stationary phase was started at a later time. We therefore assumed that the difference in the average burst size observed at 42 C at T_7 should eventually appear around T_{17}–T_{18} at 30 C. Such a difference has never been observed (see Fig. 3b). It can also be seen in Fig. 3a that at the restrictive temperature the ts-4 mutant showed a sharp drop in the average burst size at the end of exponential growth, whereas in the parental strain no drop was observed. However, at the permissive temperature this drop occurred in both strains (Fig. 3b).

Relation of average burst size to sporulation process. Stimulated by the experiments of Losick and Sonenshein (10) on the 3610 *B. subtilis* wild type and its rifampin-resistant derivative, an attempt was made to correlate, in our experiments, the drop in the average burst size with sporulation capacity. It was shown (Fig. 3a) that the ability of the φe phage to replicate in the sporulating 168tt strain is lower than that of ts-4 mutant grown at the restrictive temperature where sporulation is blocked and that no such difference exists at the permissive temperature. This observation is in agreement

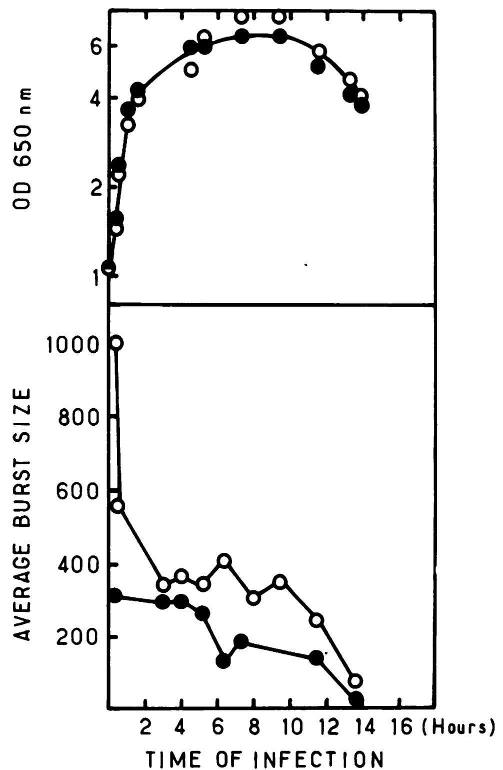

FIG. 3a. *Average burst size of phage φe after infection at various times during the growth cycle of a partially synchronized culture of Bacillus subtilis 168tt and ts-4 at 42 C. In the former culture, but not in the mutant strain, 50% refractile bodies was observed at T_7. (●) 168tt; (O) ts-4.*

with the results obtained by Losick and Sonenshein (10). However, the time during sporulation at which this difference was observed in our experiments makes any correlation with the initiation of sporulation process uncertain. It is possible that the difference observed here is only a reflection of phage trapping, which might depend upon the developmental stage of the sporulating cells. This point will be further discussed later.

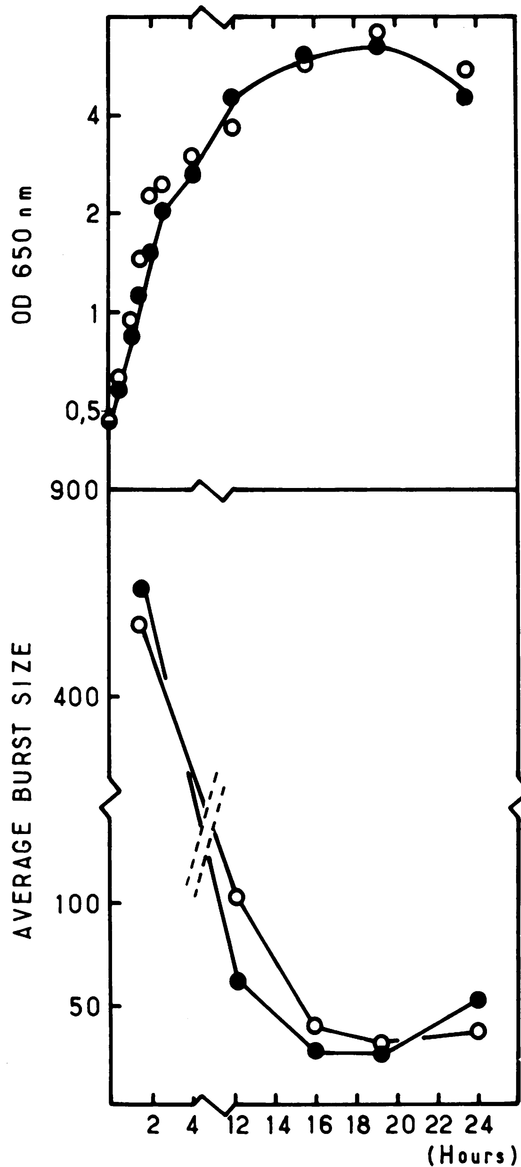

FIG. 3b. *As described in Fig. 3a, except that both strains were grown at 30 C. In this experiment, 50% refractile bodies were observed in both cultures at about* T_{22}.

Activity of the DNA-dependent RNA polymerase in the 168tt strain and the ts-4 mutant. A possible involvement of the DNA-dependent RNA polymerase in the initiation and control mechanisms of spore development has been reported by different authors (5, 6, 9, 10). This led us to examine the activity of the RNA polymerase in the thermosensitive mutant grown at the restrictive temperature. Because the present work required assays of a great number of cell samples for RNA polymerase activity, only partially purified enzyme preparations were used throughout this study. To protect these preparations against proteolytic degradation, it was necessary to learn the most suitable conditions for the isolation of this enzyme from the cells during growth cycle. Table 1 shows the specific activities of a partially purified RNA polymerase preparation (see Materials and Methods) obtained by two different methods of extraction, in the presence or absence of protease inhibitors. It can be seen that washing of the cells with buffer A containing 1 M KCl and the presence of relatively high concentrations of inhibitors increased the specific activity considerably in the presence of ϕe DNA or poly d(AT). With RNA polymerase preparations isolated from stationary-phase cells, the absence of these inhibitors throughout the isolation procedure might reduce the specific activity to about 30 to 40% of that observed when the above treatment was applied. This is also true for the change in the ratio of the specific activity of ϕe DNA/poly d(AT), which decreased by about 50% with the RNA polymerase isolated from the cells at $T_{5.5}$ when the extraction was made by method C. These results strongly suggest that the values of the ratio ϕe DNA/poly d(AT) depend on the method of extraction.

Table 2 shows the specific activities of the partially purified RNA polymerase isolated from ts-4 cells and from the parent strain, both grown either at 42 or 30 C. The activity was tested in presence of ϕe DNA and poly d(AT). Relative to the ts-4 mutant, the following two observations can be made. (i) The specific activity with ϕe DNA of the RNA polymerase isolated from cells grown at 42 C is about one-third of that observed with the enzyme from the parent strain culture grown at the same temperature or of that of the RNA polymerase from either strain grown at 30 C. (ii) With the RNA polymerase derived from ts-4 cells at $T_{5.5}$ and 42 C, a decrease was observed in the ratio of specific activity of ϕe DNA/poly d(AT) compared with that of the vegetative enzyme. Although this decrease in activity was less

TABLE 1. *Effect of the extraction method on the specific activity of the RNA polymerase*

Extraction method[a]	Sp act				ϕe DNA/poly d(AT)	
	ϕe DNA		poly d(AT)			
	Late exponential	$T_{5.5}$	Late exponential	$T_{5.5}$	Late exponential	$T_{5.5}$
A	15.9	11.1	7.9	12.2	2.1	0.91
B[b]	13.8	7.0	3.7	8.4	3.7	0.83
C	9.7	2.8	3.5	6.1	2.8	0.46

[a] Cells of *B. subtilis* 168tt were grown at 42 C in partially synchronized cultures (see Materials and Methods) and harvested at the time indicated. The cells from 400-ml cultures were treated as follows: (A) washed with 700 ml of buffer A, resuspended in buffer B (containing PMSF, DFP, and titriplex-Mg [see Materials and Methods]), and RNA polymerase was extracted by grinding the cells in a Mini-Mill (Gifford & Wood, U.S.A.) in the presence of 2.5 g of glass beads (110 μm in diameter) three times for 4 min with 2-min intervals at 0 C; (B) as in A but cells were broken by sonic disintegration for 3 min under cooling in acetone-dry ice; (C) cells were washed with only 100 ml of buffer A containing no KCl and resuspended in buffer B containing no protein inhibitors; extraction of enzyme as in B.

[b] In the later part of this work, procedure B was slightly modified, in particular by reducing the time of sonic treatment to 90 s. This led to a three- to fourfold increase in the specific activity of some of our vegetative RNA polymerase preparations (see Tables 2 and 3), including the 168tt strain (not shown in the tables).

pronounced than that with the enzyme from the parent strain grown at the same temperature, it nevertheless suggested that stationary cells from a nonsporulating mutant might have an altered RNA polymerase which transcribes ϕe template DNA with lower efficiency. This observation prompted our search for changes in the specific activity of the RNA polymerase during the growth cycle in some other asporogenic strains of *B. subtilis* blocked at stage zero of spore development. The results shown in Fig. 4 and Table 3 clearly indicate that such changes do occur. The RNA polymerase isolated from the stationary-phase cells of the Spo$^-$ mutants (12A, *trp*$^-$ prot$^-$; BS-22, *trp*$^-$ Rif^r; and BS-21, Rif^r *arg*$^-$) gradually loses its transcribing capacity of the phage ϕe DNA.

Since most of the reported work (2, 8, 9, 10) on the changes of template specificity during sporulation of *B. subtilis* was carried out with phage ϕe DNA or poly d(AT), it was certainly of some interest to investigate the transcription capacity of the *B. subtilis* RNA polymerase with *B. subtilis* DNA. The results in Table 4 show that with *B. subtilis* DNA, native or denatured, the specific activities were in all cases low compared to that obtained with ϕe DNA. The reason for this is unknown for the moment. Moreover, the results show also that with the ts-4 mutant, grown either at the permissive or restrictive temperature, there was practically no change in specific activity during growth and sporulation, whereas a sharp decrease during sporulation was observed with the 168tt strain.

TABLE 2. *Activity of RNA polymerase isolated from vegetative and sporulating cells of B. subtilis 168tt and the ts-4 mutant*

Strain	Growth temp (C)	Template DNA	Sp act			Sp act of ϕe DNA/sp act of poly d(AT)	
			Vegetative	T_2	T_5	Vegetative	T_5
ts-4	42[a]	ϕe	10.5	—	5.0		
		poly d(AT)	4.9	—	4.9	2.14	1.02
168tt	42	ϕe	32.2	8	2		
		poly d(AT)	4.9	5.3	1.15	6.57	1.74
ts-4	30[b]	ϕe	34.0	18	5		
		poly d(AT)	8.3	4	3	4.1	1.66
168tt	30	ϕe	50.0	18	5		
		poly d(AT)	5.6	3	3.5	8.93	1.43

[a] Cells were grown on single-strength nutrient broth (Difco) at 42 C (restrictive temperature). The percentage of spores (see Materials and Methods) at T_{24} was less than 0.2 with strain ts-4, whereas about 95% spores were observed in strain 168tt.

[b] When cells of both strains were grown at 30 C, a delay of about 3 to 4 h was required to reach the same percentage of refractile bodies observed in wild-type culture, at T_5 at 42 C. Therefore, at 30 C, T_2 and T_5 cells were in fact taken 6 and 9 h after the end of exponential growth.

TABLE 3. *Activity of RNA polymerase isolated from B. subtilis asporogenic strains during growth and stationary phase*

Sp^- strains	Template DNA	Sp act			Sp act of ϕe DNA/sp act poly d(AT)	
		Vegetative	T_2	T_5	Vegetative	T_5
12A (trp^-, $prot^-$)	ϕe	60	—	30.0		
	poly d(AT)	2.6		2.0	23.0	15.0
BS21 (Rif^r, arg^-)	ϕe	18.5		10.8		
	poly d(AT)	2.3		3.8	8.0	2.84
BS-22 (Rif^r, trp^-)	ϕe	41	26.6	8.8		
	poly d(AT)	3.4	3.0	2.0	12.05	4.4

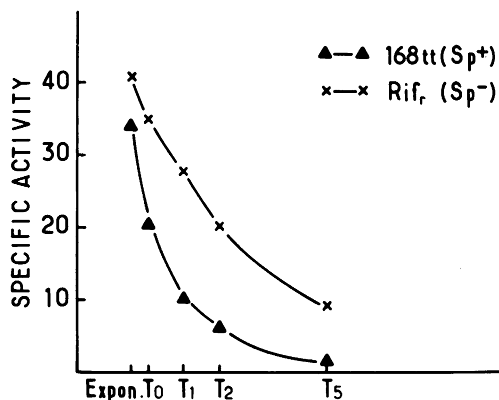

FIG. 4. *Specific activity with ϕe DNA of RNA polymerase isolated at various times during the growth cycle of Bacillus subtilis 168tt (Spo^+) and Rif^r mutant (spo^-).*

TABLE 4. *Activity of RNA polymerase isolated during growth cycle of spo^+ and spo^- strains and measured in presence of Bacillus subtilis template DNA*[a]

Strain	Growth temp (C)	RNA polymerase sp act					
		Vegetative		T_2		T_5	
		Native[b]	Denatured	Native	Denatured	Native	Denatured
168tt	42	2.78	—	1.27	—	0.27	—
	30	4.9	6.8	3.12	4.71	1.77	3.18
ts-4	42	1.0	2.1	—	—	1.2	3.1
	30	0.8	1.43	—	—	0.68	0.67
BS-22	37	1.45	2.7	0.99	2.1	0.38	1.54

[a] In this experiment, the s/c ratios (see Materials and Methods) at T_{24} were 0.9 and 0.75 for 168tt grown, respectively, at 42 and 30 C, 0.004 and 0.96 for the ts-4 mutant grown, respectively, at the restrictive and permissive temperature, and 0.01 for the BS-22, asporogenous strain.

[b] *B. subtilis* template DNA.

However, a similar drop was observed with the Rif^r Spo^- strain. These results roughly confirm those obtained with ϕe DNA (Tables 3 and 4).

Analysis by polyacrylamide gel electrophoresis of the $\beta\beta'$ subunits. The partially purified RNA polymerase preparations used in the experiments summarized in Table 2 were examined by polyacrylamide gel electrophoresis in SDS to see whether a structural modification in the β subunits takes place during sporulation. This analysis was carried out by the procedure described in Materials and Methods. Figure 5 shows that no changes in the β polypeptides were observed in RNA polymerase prepared either from samples taken up to T_{12} from cultures of 168tt grown at 42 C or from samples taken up to T_{17} from ts-4 cultures grown at 30 C. In both cases, spores were produced with high yields. These results show that under our experimental conditions no β modification was observed in the RNA polymerase of sporulating cells. Similar results were obtained by analyzing the β subunits in crude lysates (see Materials and Methods). Furthermore, these experiments also showed that the change in template specificity is independent of the structural modifications observed by others (8, 10). This question has already been discussed by one of the authors in a previous publication (21).

It should be noted that the inhibitors of proteases (PMSF, DFP, and EDTA) were added throughout the extraction and isolation of the RNA polymerase. These inhibitors were also added to the mixture for the assay of RNA polymerase activity, which is not affected by their presence. This precaution was necessary in view of the results reported previously (16), which have clearly shown that in presence of trace amounts of extracellular proteases the RNA polymerase was rapidly degraded in vitro even at 4 C. On the other hand, we have also shown (Kerjan, Millet, and Szulmajster, un-

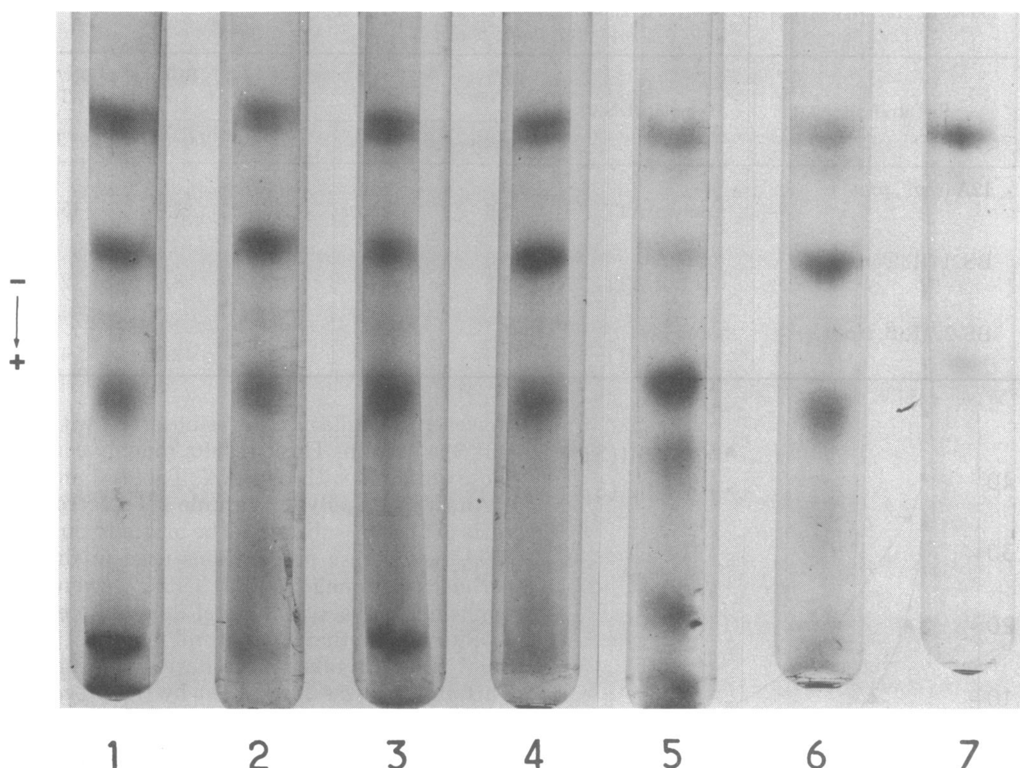

FIG. 5. *Polyacrylamide gel electrophoresis of RNA polymerase in Bacillus subtilis crude lysates (see Materials and Methods). The numbers on the bottom of each gel correspond to samples from vegetative cells (1) and sporulating cells (2, 3, 4, and 5, taken, respectively, at T_2, T_4, T_6, and T_{12}) from cultures of B. subtilis 168tt grown at 42 C. Number 6 corresponds to a T_{17} culture of the ts-4 mutant grown at the permissive temperature. Number 7 is a control and corresponds to pure core RNA polymerase isolated from B. subtilis wild type.*

published data) that, in the presence of the purified intracellular protease of *B. subtilis*, the vegetative RNA polymerase undergoes in vitro a β modification similar to that observed with the *B. megaterium* intracellular protease (16).

Intracellular protease activity in the ts-4 mutant and in the 168tt parent strain. In view of the recent studies on a possible role of proteases in the mechanism of structural modification and activity of the *B. subtilis* RNA polymerase (4, 8, 16, 21, 22), it seemed particularly important to investigate the kinetics of appearance and of the level of activity of the intracellular protease in the 168tt parent strain and in the ts-4 mutant. Figure 6 shows that the specific activity of the intracellular protease in the crude extract of this strain is at an undetectable level in the vegetative cells but increases sharply during sporulation, with a maximum at about T_9. It is noteworthy that the maximum activity in the *B. subtilis*, SMY wild-type strain was found to be at about T_3 (17).

Figure 7 shows the polyacrylamide gel electrophoretic pattern of the partially purified *B. subtilis* extracellular (a) and intracellular (b) proteases (a gift from J. Millet) and that the T_9 extract used in the experiment of Fig. 6 contained only the intracellular enzyme.

The specific activity of the intracellular protease at T_0 and T_9 in the ts-4 mutant compared with the parent strain is shown in Table 5. It is clear from this table that at 42 C there is an increase in the parent strain of about 240-fold in the specific activity of this enzyme between T_0 and T_9, whereas in the ts-4 mutant only a 6-fold increase was observed. As a consequence, one finds at T_9 in this mutant one-fifteenth as much active enzyme as in the parent strain. At the permissive temperature a similar increase in the specific activity of both strains was observed, and there was no significant difference in the levels of enzyme under these conditions. However, a correlation among the low content of the intracellular protease, the relatively weak specific activity of the RNA polymerase, and the loss of sporulation capacity in the ts-4 mutant grown at the restrictive temperature is for the moment premature.

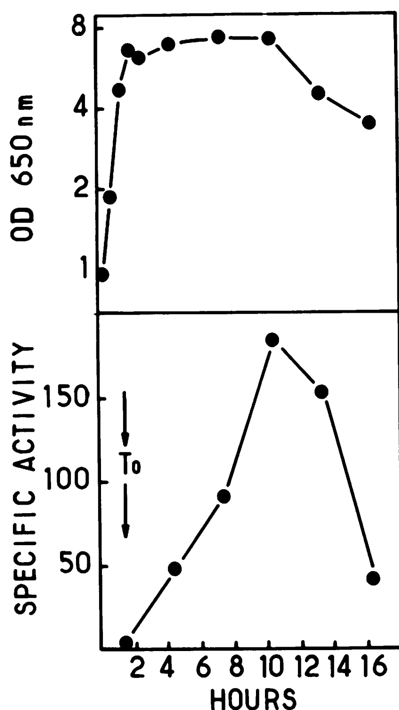

FIG. 6. *Kinetics of appearance of intracellular protease activity in crude extracts of a partially synchronized culture of Bacillus subtilis 168tt grown at 42 C (see Materials and Methods).*

DISCUSSION

The study described here was undertaken with the aim of finding out whether the thermosensitive mutation in the ts-4 mutant affects its transcription apparatus. Since this mutant, as previously described (23), is blocked at stage zero of the sporulation process, it offers the possibility of investigating the problem of initiation at the transcription level. This was done in two ways. Using the approach of Sonenshein and Roscoe (19) and Losick et al. (9, 10), we have shown that when the mutant cells were infected during stationary phase at the nonpermissive temperature, at which spore formation is arrested, the burst size of the virulent phage ϕe was about two- to threefold larger compared with that of the parent strain grown and infected at the same temperature. No such difference was observed when the cells of both strains were infected at 30 C. However, although the ts-4 mutant was found to be an early blocked mutant which should consequently lead to a difference in burst size after infection at the very beginning of the stationary phase, this difference was in fact observed about 7 h after the end of exponential growth (Fig. 3a). This observation led us to the conclusion that the

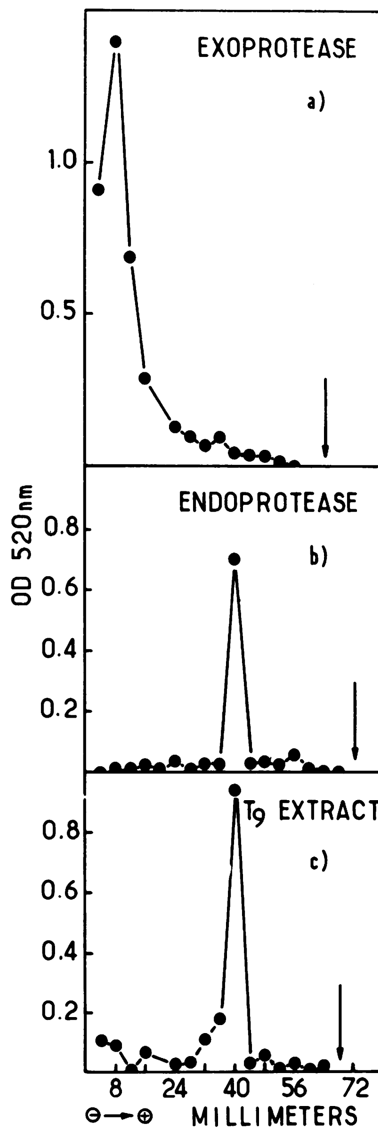

FIG. 7. *Electrophoretic pattern of polyacrylamide gels of partially purified extracellular (a) and intracellular (b) protease from Bacillus subtilis 168tt.*

TABLE 5. *Specific activity of the intracellular protease (endoprotease) of Bacillus subtilis 168tt and ts-4 from different stages of growth at 30 and 42 C*

Strain	Temp (C)	Intracellular protease (sp act)	
		T_0	T_9
B. subtilis tt	42	0.33	78
ts-4	42	0.87	5.1
B. subtilis tt	30	0.26	89.0
ts-4	30	0.19	75.4

smaller burst size in the sporulating parent strain may not be directly related to specific sporulation events but rather to phage trapping by the already made spores. It is also quite conceivable that the physiological state and metabolic activity of the bacteria greatly affect the phage burst size. That this might be the case was in fact shown by Delbrück in 1940 (3), who observed that in *E. coli*, if instead of using rapidly dividing bacteria one uses a stationary-phase culture, the burst size drops from 170 to 20.

The physiology of stationary-phase cells compared to exponential cells is, to some extent, also reflected in the in vitro experiments (Tables 2 and 3, Fig. 4; see also reference 1). In these experiments, it was shown that with the RNA polymerase from several early blocked asporogenic mutants, the specific activity in presence of ϕe template DNA decreased if the enzyme was isolated from stationary-phase cultures instead of exponentially growing cells. However, this decrease seems generally to be more pronounced and more rapid in sporulating cells. It is possible that in sporulating cells and in the stationary phase of nonsporulating cells the alterations are affecting different subunits of the RNA polymerase. But it is also possible that this difference in the two types of cells, Spo^+ or Spo^-, may only be quantitative rather than of a qualitative nature. It could well be the result of a difference in the metabolic activity of the bacteria rather than of a change in template specificity. Relative to the latter problem, it is interesting to note that conclusions by different authors (2, 8, 10) about a change in template specificity of the RNA polymerase occurring at an initial stage of the sporulation process were based only on the transcribing capacity of the *B. subtilis* RNA polymerase of either phage DNA or poly d(AT). Although it is highly possible that a change during sporulation either in the template specificity of the RNA polymerase or in the conformational state of the DNA, or both, might occur, no conclusion about these possibilities can be drawn from experiments where only phage DNA or poly d(AT) was used as template for the *B. subtilis* RNA polymerase. Even the experiments reported here (Table 4), where both the DNA template and the RNA polymerase were isolated from *B. subtilis*, show no change which could be considered specific for a sporulation event. In view of the high specific activities of the vegetative RNA polymerase in our experiments (Tables 2 and 3), the differences reported by Brevet and Sonenshein (2) concerning the transcription of ϕe DNA by the enzyme from various asporogenic mutants appear to be questionable, for their calculations were based on low specific activity measurements due to the absence of protease inhibitors during the isolation of the RNA polymerase (see Table 1). However, recent experiments (Szulmajster and Bonamy, unpublished data) have shown that the efficiency of hybridization between the RNA made in vitro by the *B. subtilis* RNA polymerase and the *B. subtilis* DNA (also used as template) is about 10 times higher than that made by the same enzyme on a ϕe DNA template. Moreover, as expected, hybridization competition experiments showed that the RNA synthesized under these two conditions differed qualitatively. All of these experiments indicate that results on transcription of phage ϕe DNA provide very limited information on the transcription of *B. subtilis* DNA and even less on gene expression during sporulation.

Concerning the ts-4 mutation, although the present study has not revealed a modification in the RNA polymerase function, we now have evidence (Szulmajster and Bonamy, unpublished data) based on DNA-RNA hybridization competition experiments that the RNA species made by the RNA polymerase from T_2-T_3 cells of the ts-4 mutant grown at the restrictive temperature is different from the RNA made by the homologous enzyme isolated from the parent strain grown at the same temperature. These experiments, therefore, would suggest that the thermosensitive mutation in the ts-4 mutant might affect the transcription mechanism.

Another important observation made in course of the work presented here concerns the modification in one of the β subunits of the RNA polymerase claimed by several investigators to occur in sporulating cells (8, 9). The experiments reported here have shown that when the protease inhibitors were added from the beginning of the extraction throughout the purification procedure no structural modification of the β subunits was observed in the enzyme isolated from sporulating cells. Therefore, we conclude that the modification observed by Losick et al. (9) and by Leighton et al. (8) is probably due to the proteolytic cleavage of the RNA polymerase in vitro. On the other hand, the finding by Maia et al. (12) of a modified β subunit in highly purified dormant spores of *B. subtilis* 168wt needs to be further clarified, since in their experiments the RNA polymerase was protected by PMSF throughout the isolation procedure, including the breakage of the spores. Moreover, under the experimental conditions described here, no intracellular protease activity has been found in the extracts

of dormant spores of *B. subtilis* (Kerjan and Szulmajster, unpublished data), and if traces of the extracellular proteases were still present a degradation of all the subunits should have been observed (16). It is possible that in dormant spores there is some proteolytic activity which is not completely inhibited by the addition of the compounds we have used. Therefore, the precise structure of the RNA polymerase in *B. subtilis* dormant spores remains to be determined.

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ADDENDUM IN PROOF

T. G. Linn, A. L. Greenleaf, R. G. Shorenstein, and R. Losick have reported (Proc. Nat. Acad. Sci. U.S.A. 70:1865-1869) that their previously described (11) structural modification of the RNA polymerase in sporulating cells wás due to proteolysis of the enzyme in vitro. This confirms our results reported here.

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