An Immunological Assay for the Sigma Subunit of RNA Polymerase in Extracts of Vegetative and Sporulating *Bacillus subtilis*

(antibody precipitation)

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ABSTRACT The activity of the σ subunit of *Bacillus subtilis* RNA polymerase decreases markedly during the first hours of sporulation [T. G. Linn *et al.* (1973) *Proc. Nat. Acad. Sci. USA* 70, 1865-1869]. We have prepared antibody against RNA polymerase holoenzyme to determine the fate of σ polypeptide during spore formation. This antiserum specifically and independently precipitates σ and core polymerase from crude extracts of *B. subtilis* as judged by both sodium dodecyl sulfate and urea gel electrophoresis of the precipitates. We report that crude extracts of sporulating cells lacking σ activity contain as much σ polypeptide as extracts of vegetative cells. However, σ polypeptide in extracts from sporulating cells is apparently only weakly associated with RNA polymerase, as indicated by the failure of σ to co-purify efficiently with core enzyme during phase partitioning.

The loss of σ activity and the weak binding of σ to core enzyme occurs normally in a mutant blocked at an intermediate stage of sporulation (*SpoII-4Z*) and in wild-type bacteria sporulating in 121B medium, Difco sporulation medium, or Sterlini-Mandelstam resuspension medium. In contrast, σ in two mutants (*SpoOa-5NA* and *SpoOb-6Z*) blocked at an early stage of spore formation remains active and tightly associated with RNA polymerase during stationary phase.

The onset of sporulation by *Bacillus subtilis* is associated with a change in the template specificity of RNA polymerase (1). Enzyme isolated from sporulating bacteria is unable to transcribe certain phage DNA templates as actively *in vitro* as RNA polymerase from vegetative *B. subtilis*. However, RNA polymerase in certain mutants blocked at an early stage of spore formation retains, throughout stationary-phase, the template recognition properties characteristic of enzyme from vegetative bacteria (2, 3). The change in template specificity is apparently caused by a marked decrease in the activity of the σ subunit of RNA polymerase rather than an alteration of the core subunits, β , β' , and α (4, 5). However, it has previously not been possible to determine whether sporulating bacteria actually lack σ polypeptide or whether the σ polypeptide is present but its activity inhibited during spore formation. To distinguish between these alternatives, we report an immunological assay that provides a direct test for the presence of the σ polypeptide in extracts of vegetative and sporulating *B. subtilis*.

METHODS

Cells. Wild-type *B. subtilis* strain NCTC 3610 (ATCC

6051), a Marburg strain, was used for all experiments except where otherwise indicated. The asporogenous mutants (6) were kindly provided by P. Schaeffer.

Media and Sporulation. Growth and sporulation of *B. subtilis* 3610 was in 121B medium (7) and sporulating cells were harvested 4.5 hr after the end of a logarithmic growth ($T_{4.5}$) unless otherwise noted. For radioactive labeling of cells, the radioactive precursor was added during early logarithmic growth. Strain SMY and the asporogenous mutants were grown (8) in Difco sporulation medium (DSM); cells were harvested either at mid-logarithmic phase or allowed to enter stationary phase by continued growth in DSM or by resuspension of logarithmically growing cells in Sterlini-Mandelstam (SM) medium (9). In DSM and SM media, sporulating or stationary-phase cells were harvested either 3 hr after the end of logarithmic growth (T_3) or 3 hr after resuspending in SM medium (T_3). After harvesting, cells were washed with buffer G (0.05 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.1 mM ethylenediaminetetraacetate, 0.1 mM dithiothreitol, 10% v/v glycerol) containing 1.0 M KCl (10) and 5% phenylmethylsulfonyl fluoride solution (6 mg/ml of 95% ethanol) to remove extracellular proteases and then rapidly frozen.

Cells Extracts Were Prepared from about 2 g of cells by disruption in a Braun homogenizer and high speed centrifugation as previously described (5) except that cells were initially suspended in buffer I (0.01 M Tris-HCl, pH 7.9, 1.0 mM ethylenediaminetetraacetate, 0.01 M $MgCl_2$, 0.3 mM dithiothreitol, and 5% phenylethylsulfonyl fluoride solution). Half the supernatant fluid was briefly sonicated to reduce viscosity and then brought to 55% saturation with solid ammonium sulfate (5). The resulting precipitate was resuspended in 1 ml of buffer I containing 0.05 M KCl and 5% v/v glycerol, dialyzed against the same buffer, and is referred to as "ammonium sulfate enzyme." The remaining half of the supernatant fluid was partitioned between phases of polyethylene glycol and dextran by a modification (11) of the procedure of Babinet (12). The final ammonium sulfate precipitate of the phase-partitioned enzyme was resuspended in 1 ml of buffer I containing 0.05 M KCl and 5% v/v glycerol and is referred to as "phase-partitioned enzyme."

Antiserum Preparation and Precipitation. The antigen for the preparation of antiserum to holoenzyme was a mixture of highly purified σ (95%) and core polymerase (5%) prepared as previously described (fraction 6 protein from ref. 11). The antigen was first covalently coupled to Sepharose 2B that

Abbreviations: SDS, sodium dodecyl sulfate; DSM, Difco sporulation medium; SM, Sterlini-Mandelstam medium.

had been activated with cyanogen bromide (13). Immunization of rabbits against the coupled antigen, purification of gamma globulin from rabbit serum, precipitation of polymerase subunits, and solubilization of the precipitates for gel electrophoresis were as previously described (5). One hundred microliters of the anti-holoenzyme antiserum were sufficient to precipitate 5.0 μg of σ and about 10.0 μg of core polymerase.

Sodium Dodecyl Sulfate (SDS) and Urea Polyacrylamide Gel Electrophoresis. High resolution SDS gels were 7.5% acrylamide in a Tris-glycine buffer (14). The urea (pH 8.7) gels were 5% acrylamide; solutions were as described by Laemmli (15) except that Tris was 2.0 M, 6 M urea replaced 0.1% SDS, and no stacking gel was used.

RESULTS

Sigma activity in extracts of vegetative and sporulating *B. subtilis*

Ammonium sulfate enzyme was prepared from vegetative and sporulating *B. subtilis* as described in *Methods* and assayed for σ activity by measuring the transcription of phage $\phi\epsilon$ DNA and poly(dA-dT) *in vitro*. Since the transcription of the phage template is largely dependent on σ , whereas transcription of the synthetic template is not, the ratio of activity with $\phi\epsilon$ DNA template to that with poly(dA-dT) serves as a measure of σ activity. The $\phi\epsilon$ to poly(dA-dT) transcription ratio for vegetative enzyme was about 5.0 (Fig. 1A), while the ratio for purified core polymerase lacking σ was about 1.0 (Fig. 1C). (Core enzyme has significant activity with $\phi\epsilon$ DNA as template even though it contains only trace amounts of σ .) In agreement with previous reports, (1, 4), sporulation ammonium sulfate enzyme displayed very little σ activity and had a $\phi\epsilon$ /poly(dA-dT) transcription ratio (Fig. 1B) similar to that of core polymerase (1.0).

An immunological assay for the σ polypeptide

To assay directly for the presence of the σ polypeptide in vegetative and sporulating extracts we prepared antiserum against vegetative holoenzyme. This antiserum independently precipitates either purified σ subunit (Fig. 2, Gel C), or core polymerase (not shown).

The antiserum was first used to precipitate RNA polymerase from the ammonium sulfate enzyme from the experiment of Fig. 1A. The precipitate was solubilized, analyzed by SDS gel electrophoresis and found to contain $\beta'\beta$ (150,000 daltons), α (43,000 daltons), antibody polypeptides and, in addition, a polypeptide with the mobility of authentic σ (55,000 daltons) (Fig. 2, Gel D). To confirm that the 55,000-dalton polypeptide was actually σ , this protein was also subjected to urea gel electrophoresis. The σ subunit migrates more rapidly than α (Fig. 2, Gels E and F) during urea gel electrophoresis, even though α migrates faster than σ during SDS gel electrophoresis (Fig. 2, Gels A and B). A slice containing putative σ subunit was cut from a duplicate SDS gel and subjected to electrophoresis on a urea gel. Gel G (Fig. 2) shows that this slice contained a polypeptide with a mobility of authentic σ and some contaminating antibody heavy chain. Thus, the antibody directed against holoenzyme specifically precipitates the σ polypeptide and the subunits of core polymerase from extracts of vegetative cells.

Next, antiserum was used to precipitate RNA polymerase from the sporulation ammonium sulfate enzyme from the

experiment of Fig. 1B. SDS gel analysis showed that the precipitate contained $\beta'\beta$, proteins of 100,000 and 70,000 daltons, α , antibody polypeptides, and apparently σ . From a densitometer tracing of Gel A in Fig. 3 we conclude that the stoichiometry of the core subunits was $\beta'\beta\alpha_2$. The 70,000-dalton protein has previously been described (16) and is a new sporulation protein bound to RNA polymerase. It is not known whether the 100,000-dalton protein is also associated with RNA polymerase. As further evidence that the 55,000-dalton polypeptide in the SDS gel was σ , this species was found to migrate with authentic σ during urea gel electrophoresis (Fig. 3, Gels B, C, and D). Thus, the σ polypeptide is present in extracts of sporulating cells and is precipitated by antiserum to holoenzyme.

FIG. 1. Transcription of phage $\phi\epsilon$ DNA and poly(dA-dT) by ammonium sulfate enzyme and phase-partitioned enzyme from vegetative and sporulating *B. subtilis*. The RNA polymerase assay was as previously described (1) except that the specific activity of [^{14}C]ATP was increased to 8 $\mu\text{Ci}/\mu\text{mole}$. Assay mixtures contained either 6 μg of $\phi\epsilon$ DNA (●) or 10 μg of poly(dA-dT) (Δ) as template and various amounts of either vegetative ammonium sulfate enzyme (A), sporulation ammonium sulfate enzyme (B), vegetative phase-extracted enzyme (D), or sporulation phase-extracted enzyme (E). The data from A and B were re-plotted in C as activity with $\phi\epsilon$ DNA versus activity with poly(dA-dT) for the vegetative (■) and the sporulation (▲) enzymes. The data in D and E were similarly re-plotted in F. The activities (□) for 160 ng, 320 ng, and 640 ng of purified core polymerase (11) with $\phi\epsilon$ DNA and poly(dA-dT) were also plotted in C and F.

FIG. 2 (left). SDS and urea polyacrylamide gel electrophoresis patterns of RNA polymerase subunits from vegetative cells. *SDS gel electrophoresis*: A, 2.6 μ g of core polymerase plus 1.0 μ g of purified σ ; B, 2.0 μ g of purified σ ; C, antibody precipitate from a mixture of 2.0 μ g of purified σ and 200 μ l of antiserum; D, antibody precipitate from a mixture of 600 μ g of the ammonium sulfate enzyme from the experiment of Fig. 1A plus 100 μ l of antiserum. *Urea gel electrophoresis*: E, 8 μ g of holoenzyme; F, a 3-mm slice containing σ cut from an unstained duplicate of SDS gel B; G, a 3-mm slice cut from the σ region of an unstained duplicate of SDS gel D. H chain is the heavy chain of the antibody.

FIG. 3 (right). SDS and urea polyacrylamide gel electrophoresis patterns of RNA polymerase subunits precipitated from sporulation ammonium sulfate enzyme by antibody. *SDS gel electrophoresis*: A, one third of the solubilized antibody precipitate from a mixture containing 550 μ g of the ammonium sulfate enzyme from the experiment of Fig. 1B plus 100 μ l of antiserum. *Urea gel electrophoresis*: B, a 3-mm slice containing σ cut from an unstained SDS gel of purified σ (same as gel F of Fig. 2); C, a 3-mm slice cut from the σ region of an unstained duplicate of SDS gel A; D, a 3-mm slice cut from the σ region of an unstained SDS gel on which a mixture of 2 μ g of purified σ and one third of the solubilized antibody precipitate of gel A had been subjected to electrophoresis. d = daltons.

To compare quantitatively the amount of σ in vegetative and sporulation extracts, ammonium sulfate enzyme was prepared from vegetative cells radioactively labeled with ^3H and sporulating cells labeled with ^{35}S . The extracts were first mixed and then precipitated with antiserum. The precipitate was solubilized and subjected to electrophoresis on an SDS gel. After staining, slices containing either β' and β or σ were

cut from the SDS gel and the radioactivity in each slice was measured. Table 1 (*Post mixing*) presents the ratio of radioactivity in the σ and $\beta'\beta$ subunits from sporulating (^{35}S) and vegetative (^3H) cells. The $^{35}\text{S}/^3\text{H}$ ratio for the $\beta'\beta$ subunits compared to the ratio for the σ subunit is a measure of the relative amount of σ in vegetative and sporulation extracts. Since $^{35}\text{S}/^3\text{H}$ for $\beta'\beta$ was 0.56 and the ratio for σ was 0.58, we conclude that there are approximately equal amounts of σ , relative to core polymerase, in both vegetative and sporulation extracts. By dividing the $^{35}\text{S}/^3\text{H}$ ratio for σ by the $^{35}\text{S}/^3\text{H}$ ratio for $\beta'\beta$ we calculate a normalized ratio of 1.06 for the amount of σ in the sporulation extract relative to the vegetative extract. Therefore, despite the low level of σ activity, sporulation extracts contain as much σ polypeptide as vegetative extracts.

Co-purification of σ with core polymerase during phase partitioning

Although the σ polypeptide is present in sporulation extracts, it was not known whether σ is actually bound to polymerase. As a test of the binding of σ to core enzyme in sporulation extracts, we determined whether σ co-purifies with RNA polymerase during partitioning of crude extracts between phases of polyethylene glycol and dextran. This procedure efficiently purifies holoenzyme from vegetative extracts (11), although the 70,000-dalton protein associated with RNA polymerase in sporulating cells does not remain bound to core enzyme during this purification procedure (17). RNA polymerase purified by phase partitioning from vegetative extracts had a $\phi\text{e}/\text{poly}(\text{dA-dT})$ transcription ratio of about 5.0 (Fig. 1D), while the transcription ratio for sporulation enzyme was about 1.0, a ratio characteristic of polymerase lacking σ (Fig. 1E). Although enzyme from the sporulation extract lacked σ activity after phase partitioning, this enzyme responded normally to added purified σ *in vitro*. (The addition of 0.5 μ g of purified σ to 10 μ g of phase-extracted sporulation enzyme increased the $\phi\text{e}/\text{poly}(\text{dA-dT})$ transcription ratio from 1.0 to 5.3.) An antibody precipitate of the partially purified vegetative enzyme contained β' , β , α , and σ in addition to antibody polypeptides (Fig. 4, Gel A). In contrast, an antibody precipitate from the phase-partitioned sporulation enzyme lacked σ , although it contained the core subunits

TABLE 1. Relative amounts of σ in extracts of vegetative and sporulating *B. subtilis*

	$\beta'\beta$ Subunits			σ Subunit			Normalized ratio
	^3H cpm	^{35}S cpm	$^{35}\text{S}/^3\text{H}$	^3H cpm	^{35}S cpm	$^{35}\text{S}/^3\text{H}$	
<i>Post mixing</i>							
Ammonium sulfate enzyme	3,390	1,910	0.56	1,710	1,001	0.58	1.06
Phase-partitioned enzyme	4,183	2,543	0.60	1,322	149	0.11	0.18
<i>Prior mixing</i>							
Phase-partitioned enzyme	2,856	2,127	0.74	1,250	899	0.72	0.98

Post mixing experiment. Ammonium sulfate enzyme and phase-partitioned enzyme were prepared from sporulating cells (2 g) labeled with 2.2 mCi of [^{35}S]methionine and vegetative cells (2 g) labeled with 4.8 mCi of [^3H]methionine. The vegetative and sporulation ammonium sulfate enzymes were mixed and RNA polymerase precipitated from a 10% portion of the mixture with 150 μ l of antiserum. RNA polymerase was similarly precipitated from a 10% portion of a mixture of the vegetative and sporulation phase-extracted enzymes with 150 μ l of anti-serum. Antibody precipitates were solubilized and subjected to electrophoresis on SDS gels. After staining, 1-mm slices containing σ and $\beta'\beta$ were cut from the gels and the radioactivity from ^{35}S and ^3H was measured. The normalized ratio was calculated from the $^{35}\text{S}/^3\text{H}$ ratios for σ and $\beta'\beta$.

Prior mixing experiment. Sporulating cells (0.3 g) labeled with 2.5 mCi of [^{35}S]methionine and harvested 10 hr after the end of logarithmic growth were mixed with vegetative cells (2.5 g) labeled with 1.2 mCi of [^3H]methionine. Phase-partitioned enzyme was then prepared from the mixture. RNA polymerase was isolated by antibody precipitation and analyzed by SDS gel electrophoresis as described above.

FIG. 4 (left). SDS polyacrylamide gel electrophoresis patterns of RNA polymerase subunits precipitated by antibody from phase-partitioned vegetative and sporulation enzymes. A, solubilized antibody precipitate from a mixture containing 260 μ g of phase-partitioned vegetative enzyme from the experiment of Fig. 1D plus 100 μ l of antiserum; B, the solubilized antibody precipitate from a mixture containing 270 μ g of phase-partitioned sporulation enzyme from the experiment of Fig. 1E plus 100 μ l of antiserum; C, the solubilized antibody precipitate from a mixture containing 230 μ g of the first polyethylene glycol supernatant fraction from the phase-extracted sporulation enzyme from the experiment of Fig. 1E plus 100 μ l of antiserum.

FIG. 5 (right). SDS polyacrylamide gel electrophoresis patterns of phase-partitioned enzyme from asporogenous mutants. Antibody precipitates were prepared by incubating 100 μ l of antiserum with 300 μ g of phase-extracted enzyme from: A, vegetative SMY grown in DSM; B, sporulating SMY grown in DSM; C, sporulating SMY in SM resuspension medium; D, stationary-phase *SpoOa-5NA* grown in DSM; E, stationary-phase *SpoOa-5NA* in SM resuspension medium; F, stationary-phase *SpoOb-6Z* grown in DSM; G, stationary-phase *SpoII-4Z* grown in DSM. The enzymes were from the experiment of Table 2.

in a stoichiometry of $\beta'\beta\alpha_2$ (Fig. 4, Gel B). However, some σ polypeptide could be detected in the first polyethylene glycol supernatant of this purification procedure for both the sporulation (Fig. 4, Gel C) and the vegetative (not shown) extracts, but we have not quantitated the amount of σ in these two cases.

To compare quantitatively the amount of σ that co-purifies with core enzyme during partial purification of RNA polymerase from vegetative and sporulating cells, extracts prepared from vegetative cells labeled with ^3H and sporulating

cells labeled with ^{35}S were phase partitioned. The phase-partitioned enzymes were then mixed and RNA polymerase was precipitated by the antiserum to holoenzyme. SDS gel analysis of the precipitate revealed that $^{35}\text{S}/^3\text{H}$ for the β subunits was 0.60, while the radioactivity ratio for σ was 0.11, giving a normalized ratio of 0.18 for the amounts of σ in the sporulation extract relative to the vegetative extract (Table 1, *Post mixing*). Thus, σ in the sporulation extract does not co-purify efficiently with core enzyme during phase partitioning.

Since σ from sporulation extracts appears to co-purify poorly with core polymerase during phase partitioning, one possibility was that the σ polypeptide is altered and cannot bind to core enzyme. Another possibility was that σ remains unaltered but is unable to bind to the core polymerase in sporulating cells. To investigate these alternatives, we mixed sporulating cells labeled with ^{35}S with an 8-fold excess of vegetative cells labeled with ^3H and partially purified RNA polymerase from an extract of the mixture by phase partitioning. RNA polymerase was precipitated from the phase-partitioned enzyme by antiserum to holoenzyme and analyzed by SDS gel electrophoresis. We found that under conditions of excess vegetative cells σ from sporulating bacteria co-purified with core polymerase as efficiently as σ from vegetative cells (Table 1, *Prior mixing*). Apparently, σ from the sporulating cells is unaltered and is not intrinsically reduced in its ability to bind to core polymerase.

The σ subunit of RNA polymerase in asporogenous mutants

RNA polymerase in certain mutants blocked at Stage 0 of sporulation fail to lose σ activity during stationary phase (2, 3). We have checked whether the σ subunit from two such mutants harvested at stationary phase co-purifies with RNA polymerase during phase partitioning. First, enzyme was prepared by both ammonium sulfate fractionation and phase partitioning. The $\phi\text{e}/\text{poly}(\text{dA-dT})$ transcription ratio for enzyme from wild-type bacteria (SMY) sporulating in either Difco sporulation medium (DSM) or Sterlini-Mandelstam (SM) resuspension medium was about 0.6, a ratio similar to that of core polymerase (Table 2). In contrast, enzyme from stationary-phase cells of a Stage 0 mutant (*SpoOa-5NA*) harvested either from DSM or SM resuspension medium retained a transcription ratio similar to that for RNA polymerase in vegetative extracts (4.0). Likewise, RNA polymerase from another Stage 0 mutant (*SpoOb-6Z*) exhibited a high $\phi\text{e}/\text{poly}(\text{dA-dT})$ transcription ratio. However, enzyme from a mutant blocked at Stage II of sporulation (*SpoII-4Z*) had undergone the change in template specificity normally. Precipitation by antiserum to holoenzyme revealed that RNA polymerase purified by phase partitioning from stationary-phase cells of the wild type and Stage II mutant contained only low amounts of σ , while phase-extracted enzyme from the early blocked mutants apparently contained normal amounts of the σ subunit (Fig. 5).

DISCUSSION

Extracts prepared from sporulating *B. subtilis* contain σ polypeptide even though RNA polymerase in these extracts displays only very low levels of σ activity. Moreover, the amount of σ subunit in extracts from sporulating cells is as great, relative to the subunits of core RNA polymerase, as

TABLE 2. Transcription ratios for RNA polymerase from asporogenous mutants

Strain	Time	Medium	Ammonium sulfate enzyme	Phase-partitioned enzyme
SMY	Veg	DSM	3.5	3.5
SMY	T ₃	DSM	0.6	0.6
SMY	T ₃	SM	0.6	0.5
<i>SpoOa-5Na</i>	T ₃	DSM	3.6	3.9
<i>SpoOa-5NA</i>	T ₃	SM	4.1	3.8
<i>SpoOb-6Z</i>	T ₃	DSM	3.9	4.0
<i>SpoII-4Z</i>	T ₃	DSM	0.8	0.6

Various amounts of either ammonium sulfate enzyme or phase-extracted enzyme prepared from each of the indicated strains of bacteria were assayed with 6 μ g of ϕe DNA or 10 μ g of poly(dA-dT) as template. The activities were plotted against protein concentration as illustrated in Fig. 1 and the $\phi\text{e}/\text{poly}(\text{dA-dT})$ transcription ratios were computed from the linear portions of the activity curves. Veg = vegetative.

the amount of σ in extracts from vegetative bacteria. However, σ polypeptide in crude extracts from sporulating cells is apparently not tightly associated with RNA polymerase, as indicated by the failure of σ to efficiently co-purify with core polymerase during phase partitioning. (It is possible, however, that σ is weakly bound to polymerase and would not be removed from the core enzyme during other purification procedures.) Thus, the lack of σ activity in sporulation extracts may be caused by the failure of σ to bind tightly to core polymerase.

The low level of σ activity in sporulation extracts and the failure of σ to bind tightly to core polymerase could be caused by an alteration of σ , an alteration of core polymerase, or a new component of sporulating cells that inhibits the binding of σ to core enzyme. It is unlikely that σ is altered, since under the conditions of the "Prior mixing" experiment of Table 1, σ from the sporulating bacteria co-purified as efficiently with RNA polymerase as σ from the vegetative bacteria. It is also unlikely that an alteration of core polymerase interferes with the function of σ , since the core subunits β' , β , and α isolated early during sporulation are apparently indistinguishable from those of vegetative enzyme and since sporulation core polymerase is stimulated to the same extent *in vitro* as vegetative core polymerase by various amounts of purified σ (T. Linn, unpublished data, and ref. 5). We therefore suggest that sporulating bacteria contain an inhibitor of σ activity and that this inhibitor acts by interfering with the binding of the σ subunit to RNA polymerase. Proof of this hypothesis will require the isolation of such an inhibitor from sporulating bacteria. It is interesting to note that Stevens (18) has recently isolated a fraction from *Escherichia coli* infected with phage T₄ that inhibits σ activity *in vitro*.

Brevet and Sonenshein (2) have shown that the loss of σ activity characteristic of RNA polymerase from wild-type cells undergoing sporulation does not occur in certain mutants blocked at Stage 0, but occurs normally in mutants that proceed to Stage II and beyond. Our finding that phase-partitioned enzyme from stationary-phase cells of two Stage 0 mutants contains σ polypeptide, while RNA polymerase from a mutant blocked at Stage II contained little or no σ is consistent with the pattern of σ activity in extracts from these mutants.

The finding that certain Stage 0 mutants retain σ activity demonstrates that the alteration of RNA polymerase is associated with events occurring early during sporulation and is not merely a consequence of stationary-phase growth. It should be noted, however, that the stage of morphological block need not correspond temporally to the loss of σ activity, since morphological events are not necessarily directly related to the time of transcription of different classes of sporulation genes. For instance, certain stage 0 mutants that partially lose σ activity (2, 10, 19) could be blocked at an event other than alteration of RNA polymerase that is required for later stages of sporulation. Similarly, the retention of σ activity by a Stage II mutant (*SpoII* 46-7, ref. 19) could indicate that loss of σ activity is not necessary to achieve Stage II of spore formation but may be required for subsequent stages of sporulation.

A preliminary report by Strauss (20) suggested that *B. subtilis* resuspended in SM sporulation medium does not undergo the loss of σ activity characteristic of bacteria

sporulating either in DSM or 121B medium. In our hands, however, RNA polymerase from bacteria sporulating in SM media lacks σ activity and RNA polymerase partially purified by phase partitioning lacks σ polypeptide, although phase-partitioned enzyme from a Stage 0 mutant resuspended in SM medium displays high levels of σ activity and contains σ polypeptide (Table 2 and Fig. 5).

It was previously suggested that the failure of phage $\phi\epsilon$ to grow on sporulating bacteria could be accounted for by the loss of σ activity, since the decrease in phage burst size during spore formation closely parallels the loss of the ability of RNA polymerase to transcribe phage $\phi\epsilon$ DNA as measured *in vitro* (1). Moreover, a sporulation-defective mutant that partially retains σ activity supports the continued growth of $\phi\epsilon$ during late stationary phase (3). This proposal has now received strong support from the finding (J. Segall, R. Tjian, J. Pero, and R. Losick, in preparation) that the rate of $\phi\epsilon$ transcription *in vivo* after infection of wild-type sporulating cells or after infection of stationary-phase cells of a Stage 0 mutant closely reflects the template specificity of RNA polymerase from these bacteria.

Note Added in Proof. Recent experiments indicate that chloramphenicol treatment of sporulating cells rapidly relieves the inhibition of σ activity as measured *in vitro* and that drug-treated sporulating bacteria support transcription by $\phi\epsilon$ more actively *in vivo* than untreated sporulating bacteria. These findings could indicate that the proposed inhibitor of σ is physiologically unstable.

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1. Losick, R. & Sonenshein, A. L. (1969) *Nature* **224**, 35-37.
2. Brevet, J. & Sonenshein, A. L. (1972) *J. Bacteriol.* **112**, 1270-1274.
3. Sonenshein, A. L. & Losick, R. (1970) *Nature* **227**, 906-909.
4. Brevet, J. (1974) *Mol. Gen. Genet.* **128**, 223-231.
5. Linn, T. G., Greenleaf, A. L., Shorestein, R. G. & Losick, R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1865-1869.
6. Ionesco, H., Michel, J., Cami, B. & Schaeffer, P. (1970) *J. Appl. Bacteriol.* **33**, 13-24.
7. Sonenshein, A. L. & Roscoe, D. (1969) *Virology* **39**, 265-276.
8. Schaeffer, P., Millet, J. & Aubert, J. (1965) *Proc. Nat. Acad. Sci. USA* **54**, 704-711.
9. Sterlini, J. M. & Mandelstam, J. (1969) *Biochem. J.* **113**, 29-37.
10. Orrego, C., Kerjan, P., Manca de Nadra, M. C. & Szulmajster, J. (1973) *J. Bacteriol.* **116**, 636-647.
11. Shorestein, R. G. & Losick, R. (1973) *J. Biol. Chem.* **248**, 6163-6169.
12. Babinet, C. (1967) *Biochem. Biophys. Res. Commun.* **26**, 639-644.
13. Porath, J., Axen, R. & Ernback, S. (1967) *Nature* **215**, 1491-1492.
14. Kamen, R., Kondo, M., Romer, W. & Weissman, C. (1972) *Eur. J. Biochem.* **31**, 44-51.
15. Laemmli, U. K. (1970) *Nature* **227**, 680-685.
16. Greenleaf, A. L., Linn, T. G. & Losick, R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 490-494.
17. Greenleaf, A. L. (1974) Ph.D. Thesis, Harvard University.
18. Stevens, A. (1974) *Biochemistry* **13**, 493-503.
19. Nishimoto, H. & Takahashi, I. (1973) "Regulation de la Sporulation Microbienne," in *Colloq. Int. Centre Nat. Rech. Sci. at Gif sur Yvette, France*, 9-10.
20. Strauss, N. (1973) "Regulation de la Sporulation Microbienne," in *Colloq. Int. Centre Nat. Rech. Sci. at Gif sur Yvette, France*, 11-13.