DORMANT SPORES OF BACILLUS THURINGIENSIS CONTAIN AN INHIBITOR OF RNA POLYMERASE[1]

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Summary: RNA polymerase activity of B. thuringiensis vegetative cells is inhibited by a substance ($1 \times 10^4 - 2 \times 10^4$ daltons) contained in dormant spore extracts. Experimental evidence indicates that the inhibitor is not DNAase, RNAase or protease because neither the template, the transcript RNA nor the enzyme is degraded. Enzyme inhibition appears to be first order with respect to concentration. Furthermore, dormant spore extracts do not exhibit RNA polymerase activity of their own on any of 6 otherwise competent templates.

The initial observations (1) regarding the inability of the RNA polymerase of sporulating cells of Bacillus subtilis to transcribe bacteriophage DNA have led to the concepts regarding alteration of template specificity and the control of sporulation. Many studies have been undertaken to eludicate the biochemical mechanism of the specificity change, and various models have been proposed, including the degradation of the β subunit (2, 3), the loss of normal sigma activity (2), and the appearance of specific RNA polymerase binding proteins (4). Although the alteration of the β subunit in the core enzyme was recently shown to be an artifact (5, 6), the situation involving sigma binding and the possible interference engendered by binding proteins (7) remain to be resolved.

In this report we present evidence of another possible mode of regulation involving inhibition of the transcription of bacteriophage DNA in extracts of dormant spores. The mechanism of action, although not defined, is consistent with the model of sigma inhibition recently proposed by Tjian and Losick (8), the RNA polymerase inhibitor suggested by Buu and Sonenshein (17) and the RNA

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polymerase inhibitor reported in mycelial extracts of Histoplasma capsulatum by Boguslawski et al. (18).

MATERIALS AND METHODS

Bacillus thuringiensis var. berliner was obtained from the ARS Culture Collection, Peoria, Illinois. Cells were maintained in modified G medium (9). For vegetative extracts, cells were harvested from early exponential cultures incubated at 30 C. When spores were desired, the concentration of yeast extract was reduced from 2% to 0.5%. Cleaned spore preparations were stored in 3-ml portions (1-3 X 10^{10} spores/ml) in distilled water at -20 C.

Vegetative cells and dormant spores were suspended in assay buffer [0.01 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.0001 M EDTA, 0.0001 M dithiothreitol (DTT), 0.002 M phenylmethyl sulfonyl fluoride (PMSF)] and broken by sonication; the spore preparations required the addition of glass beads (100 μm) for disruption. The extracts were centrifuged at 12,000 xg for 10 min to remove debris and unbroken cells. The extracts were then made 60% in $(NH_4)_2SO_4$ (0.42 g/ml) and the resulting precipitate collected by centrifugation at 15,000 xg for 15 min. The precipitate containing the RNA polymerase activity was dissolved in assay buffer without PMSF and stored in the cold. Protein was determined by the method of Lowry et al. (10).

RNA polymerase activity was assayed by a modification of the method of Klier et al. (3). The final concentration of reactants in the standard reaction mixture is given in the legend to Fig. 1. The reaction was routinely run for 30 min at 37 C. The template used was DNA from bacteriophage Q (obtained from T. L. Couch, Abbott Laboratories, North Chicago, Ill.), a lytic phage of B. thuringiensis that does not grow in sporulating cells. DNA was extracted by the method of Aurisicchio (11).

RESULTS

Initial experiments dealing with template specificity of crude RNA polymerase extracts of B. thuringiensis indicated a great disparity in specific activity of the enzyme in vegetative cells and dormant spores using bacteriophage Q DNA, B. thuringiensis DNA, B. subtilis DNA, calf thymus DNA, polyoma virus DNA and poly (dA·dT) as template; all of these templates were transcribed to some extent by vegetative extracts of B. thuringiensis whereas dormant spore extracts were unable to transcribe any of them (vegetative extract 4.10 units/mg - phage Q DNA; dormant spore extract < 0.02 units/mg - all templates). Possible explanations for the result are that either the amount of polymerase varied in the extracts or that the dormant spore contained an inhibitor of RNA polymerase. To

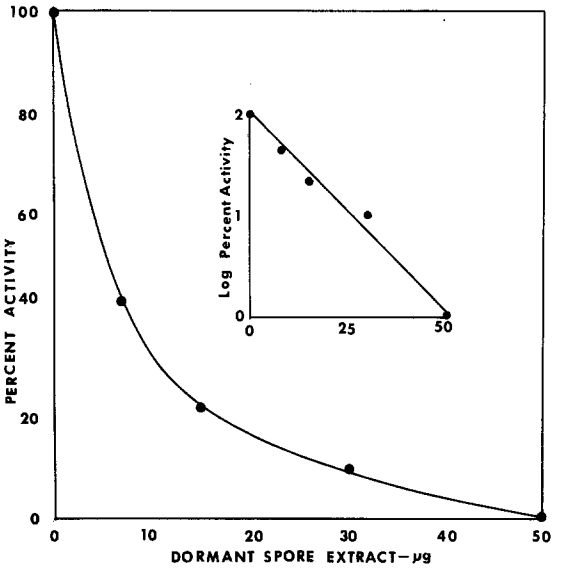

Fig. 1. Inhibition of vegetative transcription by dormant spore extracts.

The reaction mixture contained: 40 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 30 mM KCl, 0.1 mg/ml BSA, 0.4 mM GTP, ATP, CTP, 0.4 mM [5-^{3}H] UTP (sp. act. 10.0 mCi/mmole), 0.4 mM KH_2PO_4 (pH 7.5), optimum concentration of template DNA, 50 µg enzyme extract(s). The data presented are averages of duplicate samples from at least 2 preparations. The vegetative extract contained 4.10 units/mg = 100% activity. One unit of activity = 1 nmole of UMP incorporated in 30 min at 37 C.

resolve these questions, we studied the inhibitory effects of dormant spore extracts on the transcriptional activity of vegetative cell extracts. A crude extract from vegetative cells (50 µg) was titrated with increasing amounts of extract from dormant spores (Fig. 1). Greater than fifty percent inhibition (57%) of vegetative polymerase activity occurred with as little as 7 µg of the dormant spore preparation and increased to 100% when equal amounts of both extracts were present.

It is evident from these data that the dormant spore contains a potent inhibitor of RNA polymerase but does not preclude the possibility that inhibition is due to high nuclease or protease activity in the dormant spore extract. To rule out the possibility that the inhibitor activity was due to the action of a nuclease the following experiments were performed.

Nuclease activity directed against the template was examined using tritiated polyoma virus DNA (obtained from R. A. Consigli, Kansas State University) added to the standard reaction mixture (minus the radioactive nucleotide) and incubated at 37 C for 30 min. The mixture was then precipitated with 10% TCA; the precipitate was collected on a filter, washed, dried and counted. If template nuclease were present, precipitable polyoma DNA counts would have decreased. The results indicated that template integrity was not affected to a greater extent by the dormant spore lysate than the vegetative extract alone (2400 cpm [^{3}H] polyoma DNA were added: 1690 cpm were recovered with 24 µg spore and 60 µg of vegetative extract in the reaction mixture; 1520 cpm were recovered with 60 µg vegetative extract alone in the reaction mixture). The template activity of Polyoma DNA was identical to phage Q DNA. To exclude nuclease activity directed against the transcript RNA, dormant spore extract and actinomycin D were added to standard reaction mixtures containing vegetative extract. Additions were made either at the beginning or after 15 minutes (Table 1). If transcript nuclease were present in the dormant spore extract, a decrease in TCA-insoluble counts would have occurred on further incubation, after the addition of actinomycin D and the dormant spore extract. However, no such decrease occurred and from these experiments we conclude that nucleases are not responsible for inhibition of vegetative extract by dormant spore extract.

To determine whether dormant spore extracts contained a protease that altered the activity of RNA polymerase in the vegetative extracts, we preincubated a mixture of the dormant spore and vegetative extracts for 60 min at 37 C. During that time, samples were removed at intervals and assayed for transcription. If a protease were present the activity would have steadily decreased as a result of protealysis of the enzyme. However, we found that this phenomenon did not occur (Table 2); enzyme activity remained at the level of inhibition (25%) consistent with the amount of dormant spore extract added. Moreover, inhibition was immediate and persisted throughout the experiment.

Table 1. Stability of transcript RNA in the presence of dormant spore extract.

	cpm recovered
Vegetative extract alone	5110
*Veg + DS + AcD at t_{15}	2610
Veg + AcD at t_{15}	2590
Veg + DS at t_o	0
Veg + AcD at t_o	0

Standard reaction mixture contained (as appropriate) 11 µg vegetative extract, 36 µg dormant spore extract, and 10 µg actinomycin D. Actinomycin D and the dormant spore extract were added initially or after 15 min of incubation at 37 C and incubation was continued for an additional 15 min.

*Veg = Vegetative extract; DS = dormant spore extract; AcD = actinomycin D.

Table 2. Transcription activity of preincubated vegetative and dormant spore extract.

Vegetative + dormant spore extracts (minutes of preincubation)(1)	Percent vegetative activity remaining(2)	Specific Activity units/mg
0	76	2.8
15	84	3.1
45	73	2.7
60	76	2.8

(1) The preincubation mixture consisted of 240 µg of vegetative extract and 24 µg of dormant spore extract in assay buffer minus PMSF.

(2) The specific activity of the vegetative extract (3.7 units/mg) represented 100%.
Reaction mixtures were formulated as in Fig. 1.

Partial characterization of the inhibitor substance has also been achieved. It appears to be at least, in part, protein because inhibition could be relieved by pronase or trypsin digestion. Dormant spore extract was incubated at 37 C for 2 hrs with predigested (30 min at 37 C) pronase (1.5 mg/ml in incubation mixture) and subsequently boiled to destroy pronase activity. Boiling had no effect on inhibitor activity. The pronase-treated dormant spore extract was then assayed for inhibitory activity against vegetative extract transcription (vegetative extract alone, 4.3 units/mg; vegetative plus dormant spore extract, 0.62 units/mg; vegetative plus pronase-treated

dormant spore extract, 4.3 units/mg - increase in protein due to pronase was subtracted before specific activity was calculated). All reaction mixtures contained 60 µg vegetative extract and 24 µg of dormant spore extract. The identical experiment using trypsin protealysis produced the same result.

The molecular weight range of the active fraction was determined by anisotropic ultrafiltration with an Amicon ultrafiltration cell. Inhibitor activity passed through the 20,000 dalton membrane but was retained by the 10,000 dalton membrane. Therefore, the molecular weight appears to be between 10,000 and 20,000 daltons. Further purification and characterization of the inhibitor are in progress.

DISCUSSION

We have presented evidence for a potentially important inhibitor of RNA polymerase activity in B. thuringiensis. A substantial excess of the inhibitor is present in dormant spores because these extracts not only inhibit vegetative polymerase but also continue to show no activity of their own. The mode of inhibition is apparently first order (Fig. 1) with respect to concentration suggesting perhaps a single step inhibition of the reaction. The loss of RNA polymerase activity is not due to DNAase, RNAase or protease, but apparently to a substance of molecular weight 10,000-20,000 daltons. The data we have presented leads us to believe that the material in question is a protein, however other macromolecules such as t-RNA have not been specifically eliminated.

Although others have reported the presence of inhibitors in sporulating cells, the inactivation was either unique to those cells because the spore enzyme could not transcribe a phage genome (17) or was irreversible (14). Not only are the dormant spore extracts of B. thuringiensis unable to transcribe phage Q DNA but they also are unable to transcribe five other competent templates. Whether such activity is related to the template

specificity changes attendent to sporulation or to the maintainence of enzyme conformation in the absence of water during dormancy is unknown. Additional data (unpublished) also indicate that the inhibitor is inactivated during spore outgrowth resulting in the reappearance of RNA polymerase activity. The recent work of Buu and Sonenshein (17) which suggests an interfering component that they refer to as "sigma inhibitor" bears some similarities to our system in that inhibition is relieved after 40 min of outgrowth. However, the significant difference between their results and ours regards enzyme activity. We have found no RNA polymerase activity using poly (dA·dT) with the dormant spore extract alone or when used to inhibit vegetative extracts. The lack of inhibitor specificity for either the spore or vegetative polymerase as well as the lack of poly (dA·dT) transcription argues against a sigma effect. Consequently, we conclude that the interfering component in B. thuringiensis does not function by arresting sigma activity.

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