Chloramphenicol Restores Sigma Factor Activity to Sporulating *Bacillus subtilis*

(transcription by phage ϕ e/sigma subunit of RNA polymerase)

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ABSTRACT The σ subunit of RNA polymerase from sporulating *Bacillus subtilis* is markedly inhibited in its ability to direct active transcription of phage ϕ e DNA *in vitro*. Treatment of sporulating bacteria with chloramphenicol rapidly restores σ activity, suggesting that sporulating cells contain an inhibitor of σ that is physiologically unstable or that becomes unstable after drug treatment. The hypothetical inhibitor is depleted exponentially with an apparent half-life of 11 min at 37°.

The onset of sporulation by *Bacillus subtilis* is associated with a marked decrease in the ability of the σ subunit of RNA polymerase to direct active transcription of phage ϕ e DNA *in vitro* (1, 2). Although sporulating bacteria contain as much σ polypeptide as vegetative bacteria, σ from sporulating *B. subtilis* is functionally inhibited and only weakly associated with RNA polymerase (3). It was previously observed that *B. subtilis* phage ϕ e is unable to grow in sporulating bacteria (4) and that the time course of decrease in phage burst size during the first hours of spore formation closely parallels the decrease in σ activity, as indicated by the reduced ability of RNA polymerase to transcribe ϕ e DNA *in vitro* (5). In contrast, a sporulation-defective mutant of *B. subtilis* that partially retains σ activity supports ϕ e growth during late stationary phase (6).

To test the idea that the failure of ϕ e to grow in wild-type sporulating cells is related to the inhibition of σ activity (5), we recently undertook an investigation of the relative rate of ϕ e transcription after infection of vegetative and sporulating *B. subtilis*. In agreement with the idea that the inhibition of σ prevents the growth of ϕ e we found, as reported here, that ϕ e DNA is transcribed only 9% as actively *in vivo* in early

sporulating cells as in vegetative bacteria. During the course of this study it was unexpectedly discovered that sporulating bacteria briefly treated with chloramphenicol, a drug that blocks protein synthesis, actively support phage transcription. This novel response suggested that drug treatment had depleted sporulating bacteria of an unstable inhibitor of σ activity. In support of this idea we report that chloramphenicol treatment of sporulating cells rapidly restores σ activity as measured *in vitro*.

Rate of ϕ e transcription

To compare the rates of ϕ e DNA transcription in vegetative and sporulating bacteria, we first compared the rates of total cellular RNA synthesis two minutes after phage infection. Sporulating cells were infected at 2 hr after the end of logarithmic growth (T_2 of sporulation), by which time infection by ϕ e leads to a burst size reduced 10-fold in comparison with that from vegetative cells (4, 5). Calculating total RNA synthesis from the incorporation of [³H]uridine into RNA and the labeling of the pool of nucleoside triphosphates (Fig. 1), we found that the rates of total RNA synthesis after infection of vegetative and sporulating bacteria were approximately 0.95 and 0.17 nmol/min per 10⁸ cells, respectively (Table 1). Next we determined the fraction of RNA labeled at 2-4 min after infection that hybridized to denatured ϕ e DNA (Fig. 2A). About 22% of the pulse-labeled RNA from infected vegetative cells and about 11% of the radioactive RNA from infected sporulating bacteria annealed to ϕ e DNA. From the rates of total RNA synthesis, and correcting for the efficiency of hybridization, we calculate that the rate of ϕ e transcription in sporulat-

TABLE 1. Rates of ϕ e transcription

	% Input hybridized to		Rate of RNA synthesis (nmol of UMP/min per 10 ⁸ cells)		Relative rate of ϕ e RNA synthesis, T_2 /veg (%)
	H strand DNA	ϕ e DNA	Total RNA	ϕ e RNA	
Veg	39	22	0.95	0.312	9
T_2	51	11	0.17	0.028	
Veg, CAM	66	9.7	1.15	0.166	40
T_2 , CAM	32	28	0.16	0.067	

The RNA extracted from the infected and pulse-labeled cells of the experiments of Fig. 2A and B was hybridized to heavy (H) strand DNA of *B. subtilis* and to denatured ϕ e DNA (Fig. 2) and the % input hybridized at near saturating amounts of DNA was determined. The rates of total cellular RNA synthesis were estimated from the initial slopes of the curves of Fig. 1D (the data are the average of two independent experiments). The rate of ϕ e RNA synthesis is the product of the rate of total RNA synthesis and the % input hybridized to ϕ e DNA corrected for the efficiency of hybridization of ϕ e RNA to denatured ϕ e DNA (67%, unpublished results). For comparison the rates of total RNA synthesis in uninfected vegetative (veg) and sporulating (T_2) cells were 1.29 and 0.25 nmol UMP/min per 10⁸ cells, respectively.

CAM is chloramphenicol.

FIG. 1. Rates of total RNA synthesis. After at least 10 generations of growth in 121B medium (4) containing 0.4 mM [32 P]-phosphate (70 Ci/mol), 1 ml of vegetative ($\square$) or 1 ml of sporulating (∇) cells were infected with ϕ (multiplicity of infection = 5) and labeled at 37° with 100 μ Ci of [3 H]uridine (28 Ci/mmol) at 2 min after infection. Similarly, vegetative ($\blacksquare$) and sporulating ($\blacktriangledown$) cells that had been treated for 20 min with chloramphenicol (150 μ g/ml) were also infected with ϕ and labeled with [3 H]uridine. Nucleotides were extracted from 100- μ l samples at the indicated times with 4 M formic acid and resolved by thin-layer chromatography (7). Approximately 85% of the 3 H was in UTP and the remaining 3 H was in CTP. The specific activity of UTP (A) and CTP (B) was calculated from the 3 H/ 32 P ratio for each nucleotide and the specific activity of [32 P]phosphate in the medium (7). (C) Incorporation of [3 H]uridine into RNA during each time interval was determined by precipitating 5- μ l samples with cold trichloroacetic acid solution. (Greater than 95% of the trichloroacetic-acid-insoluble 3 H was alkali labile.) (D) The incorporation of UMP into RNA during each time interval was calculated from the following relation as derived from the discussion of Lazzarini and Dahlberg (7): UMP in RNA = [3 H]RNA/[UTP + (y/x)CTP], where [3 H]RNA is the radioactivity incorporated into RNA during the time interval, UTP and CTP are the average specific activities of UTP and CTP over the time interval, and x and y are the proportions of uridine and cytidine, respectively, in the pulse-labeled RNA. The values for x and y range from 24.2 to 28.3 and 20.1 to 20.8 mol %, respectively, for pulse-labeled RNAs from various growth phases of *B. subtilis* (8). Using an average value of 0.78 for the y/x ratio, we approximated the increment of UMP incorporated into RNA during each time interval. [Since CTP was much smaller than UTP, differences in the y/x ratio between the vegetative and sporulation RNAs would have introduced only a small error (<3%).] The time course of RNA synthesis was then obtained by summation of these increments (9).

ing cells (0.028 nmol/min per 10^8 cells) was 9% of the rate (0.31 nmol/min per 10^8 cells) in vegetative bacteria (Table 1).

It is known that ϕ and the closely related phage SPO1 induce new RNA polymerase-binding proteins (12) that could enhance the rate of phage transcription. To measure the rate of ϕ RNA synthesis in the absence of phage protein synthesis, vegetative and sporulating bacteria were treated with chloramphenicol for 20 min before infection by ϕ . Although drug treatment did not significantly affect the rate of total RNA synthesis (Fig. 1 and Table 1), the proportion of pulse-labeled

FIG. 2. Hybridization of ϕ RNA from infected vegetative and sporulating *B. subtilis*. (A) Growth and sporulation was at 37° in 121B medium (4) containing 0.4 mM phosphate. One milliliter of vegetative cells (2.4×10^8) was labeled with 100 μ Ci of [3 H]uridine (28 Ci/mmol) at 2–4 min after infection with ϕ (multiplicity of infection = 5). After the pulse, the cells were rapidly chilled and incubated for 15 min at 0° in 2 ml of protoplast buffer (10) containing 160 μ g of lysozyme per ml. The protoplasts were then frozen over dry ice-acetone. One ml of sporulating (T_2) cells (5.8×10^8) was similarly labeled with 0.8 mCi of “carrier-free” [32 P]phosphate at 2–4 min after infection with ϕ (multiplicity of infection = 5). The frozen protoplasts of the infected sporulating cells were mixed with the vegetative protoplasts in 0.5 ml of lysing buffer (10) and heated at 95° for 2 min. After incubation of the lysate with 15 μ l of diethylpyrocabonate at 37° for 5 min, the RNA was extracted and precipitated as described (10) and then resuspended in 0.5 ml of $2 \times$ SSC (0.3 M NaCl, 0.03 M sodium citrate), pH 7.4. The RNA was then hybridized to the indicated quantities of heat-denatured ϕ DNA in 0.125 ml of $2 \times$ SSC, pH 7.4, for 3.5 hr at 68° (11). The reaction mixtures were then incubated with 0.8 ml of heat-treated pancreatic ribonuclease solution (20 μ g/ml of $2 \times$ SSC) for 30 min at 34° and the hybrids were collected on nitrocellulose filters. The input RNA was the ribonuclease-sensitive radioactivity (96% of the 3 H and 85% of the 32 P input). The radioactivity retained on filters in the absence of ϕ DNA was subtracted as background (3% of the [3 H]RNA input and 8% of the [32 P]RNA input). $\square$, Vegetative RNA (input 70,000 cpm 3 H); ∇ , sporulation RNA (input 11,000 cpm 32 P). (B) Is the same as (A) except that chloramphenicol (150 μ g/ml) was added to the cells 20 min prior to infection. $\blacksquare$, Vegetative RNA (input 11,000 cpm 3 H); $\blacktriangledown$, sporulation RNA (input 71,000 cpm 32 P).

RNA from infected vegetative cells that hybridized to denatured ϕ DNA decreased from 22% (Fig. 2A) to 9.7% (Fig. 2B). Surprisingly, however, the proportion of ϕ -specific RNA in infected sporulating cells increased from 11% (Fig. 2A) to 28% (Fig. 2B) with chloramphenicol treatment. Table 1 indicates that the rate of ϕ transcription in drug-treated sporulating cells (0.067 nmol/min per 10^8 cells) was 40% of the rate in chloramphenicol-treated vegetative bacteria (0.166 nmol/min per 10^8 cells) and 2.4 times the rate in untreated sporulating cells (0.028 nmol/min per 10^8 cells). The time course experiment of Fig. 3A shows that the proportion of ϕ -specific RNA in sporulating cells increased with the length of time that the sporulating bacteria had been exposed to chloramphenicol prior to infection.

Chloramphenicol restores σ activity

The novel effect of chloramphenicol on ϕ transcription suggested that drug treatment of sporulating bacteria had relieved the inhibition of σ activity. To test this possibility RNA polymerase was purified partially by phase partitioning or ammonium sulfate fractionation from untreated and drug-

FIG. 3. Time course of the effect of chloramphenicol on ϕ_e transcription and σ activity. (A) Vegetative and sporulating cells that had been treated with chloramphenicol (150 $\mu\text{g/ml}$) for various times were infected with ϕ_e and pulse-labeled at 2–4 min after infection as described for Fig. 2B. RNA was extracted from the pulse-labeled cells and hybridized to various amounts of denatured ϕ_e DNA. The percent input hybridizing at near saturating amounts of DNA is plotted for vegetative [^3H]RNA (■) and sporulation [^{32}P]RNA (▼). (B) RNA polymerase was prepared by phase partitioning (3) from sporulating cells treated with chloramphenicol (150 $\mu\text{g/ml}$) for various times. The $\phi_e/\text{poly(dA-dT)}$ transcription ratios were determined as described for Fig. 4 and Table 2. (C) Percent inhibition of σ activity was calculated from the data in panel B as described in the text and is plotted on a logarithmic scale.

treated vegetative and sporulating cells and assayed with either ϕ_e DNA or poly(dA-dT) as templates (Fig. 4 and Table 2). Since transcription of the phage DNA is largely dependent on σ , while transcription of the synthetic template is somewhat depressed by σ , the ratio of activity with these two DNAs serves as a measure of σ activity (13). The $\phi_e/\text{poly(dA-dT)}$ transcription ratio for phase-partitioned enzyme from vegetative cells was 5.2 (Fig. 4A) while the ratio for sporulation polymerase, 1.1 (Fig. 4B), was similar to that for purified core RNA polymerase lacking σ (3). In contrast, phase-partitioned RNA polymerase from sporulating cells that had been treated with chloramphenicol for 20 min before harvesting exhibited a ratio of 4.0 (Fig. 4C). The increase in transcription ratio caused by chloramphenicol was also observed for polymerase from sporulating cells that had been infected by ϕ_e after drug treatment (Table 2).

TABLE 2. Transcription ratios for RNA polymerase

Purification	Drug	Phage infection	$\phi_e/\text{poly(dA-dT)}$ ratio	
			Veg	T ₂
Partitioned Enzyme	—	—	5.2	1.1
Partitioned Enzyme	CAM	—	5.0	4.0
Partitioned Enzyme	Str	—	—	4.0
(NH ₄) ₂ SO ₄ Enzyme	—	—	5.0	1.0
(NH ₄) ₂ SO ₄ Enzyme	CAM	—	—	3.0
(NH ₄) ₂ SO ₄ Enzyme	—	ϕ_e	4.2	1.4
(NH ₄) ₂ SO ₄ Enzyme	CAM	ϕ_e	3.5	3.4

RNA polymerase was prepared either by ammonium sulfate fractionation (3) or phase partitioning (3). Enzyme activities were plotted against protein concentration as illustrated in Fig. 4 and the $\phi_e/\text{poly(dA-dT)}$ transcription ratios were computed from the linear portion of the activity curves. Chloramphenicol (CAM) and streptomycin (Str) treatments (150 $\mu\text{g/ml}$) were for 20 min before harvesting. Infected cells (multiplicity of infection = 5) were harvested 3–4 min after ϕ_e infection.

The time course experiment of Fig. 3B indicates that the transcription ratio increased with the length of time that the sporulating cells had been treated with chloramphenicol; the ratio for polymerase from cells treated for 12 min was 3.2 while the ratio for polymerase from 30-min treated bacteria, 4.5, was almost as high as that for vegetative RNA polymerase. The effect of chloramphenicol is not due to direct interaction with RNA polymerase, since the addition of chloramphenicol *in vitro* to sporulation enzyme (prepared by ammonium sulfate fractionation) did not increase the $\phi_e/\text{poly(dA-dT)}$ transcription ratio (data not shown). The effect of drug treatment on sporulating cells is not unique to chloramphenicol, since another drug that blocks protein synthesis, streptomycin, also relieved the inhibition of σ activity (Table 2).

An investigation (3) of the inhibition of σ activity during spore formation showed that σ appears to be only weakly associated with core RNA polymerase. This weakened binding was indicated by the failure of σ to co-purify efficiently with RNA polymerase during phase partitioning of extracts of sporulating cells, a procedure that efficiently purifies holoenzyme from vegetative extracts. We employed a recently described (3) immunological assay for σ to test whether chloramphenicol treatment of sporulating bacteria had restored tight binding of σ to core enzyme. RNA polymerase was precipitated from the phase partitioned enzymes of the experiment of Fig. 4 by antiserum prepared against *B. subtilis* holoenzyme. The precipitates were then subjected to sodium dodecyl sulfate gel electrophoresis. Polymerase purified by phase partitioning from vegetative bacteria contained σ (gel insert, Fig. 4A) while sporulation phase partitioned enzyme was deficient in σ polypeptide (gel insert, Fig. 4B). The gel insert in Fig. 4C shows that phase partitioned polymerase from sporulating cells that had been treated with chloramphenicol contained significantly more σ than the enzyme from the untreated sporulating cells.

Depletion of σ inhibitor

A recent investigation into the mechanism of σ inhibition during spore formation suggested that sporulating *B. subtilis* contains an inhibitor of σ and that such an inhibitor could act by interfering with the binding of σ to core RNA polymerase

Fig. 4. Transcription of ϕ_e DNA and poly(dA-dT) by RNA polymerase from vegetative, sporulating, and chloramphenicol-treated sporulating *B. subtilis*. Growth and sporulation was at 37° in 121B medium (4). RNA polymerase was prepared by phase partitioning as previously described (3) from: (A) vegetative bacteria; (B) sporulating bacteria, (C) sporulating bacteria that had been treated with chloramphenicol (150 μ g/ml) for 20 min. RNA polymerase was assayed (5) with [14 C]ATP (8 Ci/mol) and 6 μ g of ϕ_e DNA (●) or 10 μ g of poly(dA-dT) (Δ) as templates. RNA polymerase was precipitated from 270 μ g of each phase-partitioned enzyme with 100 μ l of the antiserum against *B. subtilis* RNA polymerase holoenzyme of ref. 3 and subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide (7.5%) gels (3), shown as inserts.

(3). The finding that chloramphenicol treatment of sporulating cells rapidly relieves the inhibition of σ activity and restores tight binding of σ to core polymerase is most easily explained by proposing that the inhibitor of σ is physiologically unstable and is rapidly depleted *in vivo* in the absence of protein synthesis. A half-life for decay of the hypothetical inhibitor can be calculated from the time course experiment of Fig. 3B by assuming that the ϕ_e /poly(dA-dT) transcription ratio of 1.0 characteristic of core polymerase (3) lacking σ represents 100% inhibition while the ratio of 5.2 for polymerase from vegetative cells (Fig. 4A) represents 0% inhibition. Fig. 3C indicates that the inhibitor decays exponentially *in vivo* with an apparent half-life of 11 min at 37°. [This half-life is considerably shorter than that for turnover of total cellular protein (4–6 hr) or turnover of the β subunits of RNA polymerase (5 hr) during sporulation (14, 15).] Another physiologically unstable protein, the product of the positive regulatory gene *N* of phage λ , is thought to interact with *Escherichia coli* RNA polymerase (16, 17) and decays *in vivo* with a half-life of 5 min at 35° (18).

Another equally plausible possibility is that the inhibitor of σ is not ordinarily unstable but that chloramphenicol treatment directly, or indirectly, causes the inhibitor to be functionally unstable and rapidly depleted in sporulating cells. A test of these hypotheses will first require the isolation of the inhibitor from sporulating *B. subtilis*. Although we favor the

view that the inhibitor is a sporulation protein, possibly a new RNA polymerase-binding protein (19), it is not excluded that the inhibitor is a nonprotein component of sporulating bacteria whose synthesis or stability is affected by chloramphenicol.

Our findings indicate that chloramphenicol treatment of sporulating cells relieves the inhibition of σ activity as measured *in vitro* and that drug-treated sporulating bacteria support transcription by ϕ_e more actively *in vivo* than untreated cells. This correlation supports the view that the inhibition of σ activity during spore formation is at least in part responsible for the low rate of ϕ_e transcription in sporulating bacteria. As a further test of this idea, we have measured the rate of ϕ_e transcription in stationary phase cells of an asporogenous mutant, *LS 3* (6, 20), that retains σ activity. We (manuscript in preparation) find that ϕ_e is transcribed about 2.6-fold more actively in early stationary phase cells of the mutant than in wild-type sporulating bacteria, although the rates of total RNA synthesis are similar in both cases.

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1. Linn, T. G., Greenleaf, A. L., Shorenstein, R. G. & Losick, R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1865–1869.
2. Brevet, J. (1974) *Mol. Gen. Genet.* **128**, 223–231.
3. Tjian, R. & Losick, R. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2872–2876.
4. Sonenshein, A. L. & Roscoe, D. H. (1969) *Virology* **39**, 265–276.
5. Losick, R. & Sonenshein, A. L. (1969) *Nature* **224**, 35–37.
6. Sonenshein, A. L. & Losick, R. (1970) *Nature* **227**, 906–909.
7. Lazzarini, R. A. & Dahlberg, A. E. (1971) *J. Biol. Chem.* **246**, 420–429.
8. Doi, R. H. & Igarashi, R. (1964) *Proc. Nat. Acad. Sci. USA* **52**, 755–762.
9. Gallant, J. & Margason, G. (1972) *J. Biol. Chem.* **247**, 2289–2294.
10. Summers, W. C. (1970) *Anal. Biochem.* **33**, 459–463.
11. Gillespie, D. & Spiegelman, S. (1965) *J. Mol. Biol.* **12**, 829–842.
12. Fox, T. D. & Pero, J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2761–2765.
13. Shorenstein, R. G. & Losick, R. (1973) *J. Biol. Chem.* **248**, 6163–6169.
14. Mazzara, G. P. (1974) Honors thesis, Harvard University.
15. Spudich, J. A. & Kornberg, A. (1968) *J. Biol. Chem.* **243**, 4600–4605.
16. Georgopoulos, C. P. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2977–2981.
17. Ghysen, A. & Pironio, M. (1972) *J. Mol. Biol.* **65**, 259–272.
18. Schwartz, M. (1970) *Virology* **40**, 23–33.
19. Greenleaf, A. L., Linn, T. G. & Losick, R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 490–494.
20. Sonenshein, A. L., Cami, B., Brevet, J. & Cote, R. (1974) *J. Bacteriol.* **120**, 253–265.