Chloramphenicol Restores Sigma Factor Activity to Sporulating Bacillus subtilis

(transcription by phage $\phi e$/sigma subunit of RNA polymerase)

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ABSTRACT The $\sigma$ subunit of RNA polymerase from sporulating Bacillus subtilis is markedly inhibited in its ability to direct active transcription of phage $\phi e$ DNA in vitro. Treatment of sporulating bacteria with chloramphenicol rapidly restores $\sigma$ activity, suggesting that sporulating cells contain an inhibitor of $\sigma$ 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 $37^\circ$.

The onset of sporulation by Bacillus subtilis is associated with a marked decrease in the ability of the $\sigma$ subunit of RNA polymerase to direct active transcription of phage $\phi e$ DNA in vitro (1, 2). Although sporulating bacteria contain as much $\sigma$ polypeptide as vegetative bacteria, $\sigma$ from sporulating B. subtilis is functionally inhibited and only weakly associated with RNA polymerase (3). It was previously observed that B. subtilis phage $\phi e$ 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 $\sigma$ activity, as indicated by the reduced ability of RNA polymerase to transcribe $\phi e$ DNA in vitro (5). In contrast, a sporulation-defective mutant of B. subtilis that partially retains $\sigma$ activity supports $\phi e$ growth during late stationary phase (6).

To test the idea that the failure of $\phi e$ to grow in wild-type sporulating cells is related to the inhibition of $\sigma$ activity (5), we recently undertook an investigation of the relative rate of $\phi e$ transcription after infection of vegetative and sporulating B. subtilis. In agreement with the idea that the inhibition of $\sigma$ prevents the growth of $\phi e$ we found, as reported here, that $\phi e$ 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 $\sigma$ activity. In support of this idea we report that chloramphenicol treatment of sporulating cells rapidly restores $\sigma$ activity as measured in vitro.

Rate of $\phi e$ transcription

To compare the rates of $\phi e$ 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 (T1 of sporulation), by which time infection by $\phi e$ 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 [H]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 10^8 cells, respectively (Table 1). Next we determined the fraction of RNA labeled at 2-4 min after infection that hybridized to denatured $\phi e$ 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 $\phi e$ DNA. From the rates of total RNA synthesis, and correcting for the efficiency of hybridization, we calculate that the rate of $\phi e$ transcription in sporulating

**Table 1. Rates of $\phi e$ transcription**

<table>
<thead>
<tr>
<th>H strand DNA</th>
<th>$\phi e$ DNA</th>
<th>Total RNA</th>
<th>$\phi e$ RNA</th>
<th>T1/veg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veg</td>
<td>39</td>
<td>22</td>
<td>0.95</td>
<td>0.312</td>
</tr>
<tr>
<td>T1</td>
<td>51</td>
<td>11</td>
<td>0.17</td>
<td>0.028</td>
</tr>
<tr>
<td>Veg, CAM</td>
<td>66</td>
<td>9.7</td>
<td>1.15</td>
<td>0.166</td>
</tr>
<tr>
<td>T1, CAM</td>
<td>32</td>
<td>28</td>
<td>0.16</td>
<td>0.067</td>
</tr>
</tbody>
</table>

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 $\phi e$ 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. 1(D) and the data are the average of two independent experiments. The rate of $\phi e$ RNA synthesis is the product of the rate of total RNA synthesis and the % input hybridized to $\phi e$ DNA corrected for the efficiency of hybridization of $\phi e$ RNA to denatured $\phi e$ DNA (67%, unpublished results). For comparison the rates of total RNA synthesis in uninfected vegetative (veg) and sporulating (T1) cells were 1.29 and 0.25 nmol UMP/min per 10^8 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 [3H]-phosphate (70 Ci/mol), 1 ml of vegetative (C) or 1 ml of sporulating (T) cells (V) were infected with $\phi$ (multiplicity of infection = 5) and labeled at 0.3 for 20 min with chloramphenicol (150 $\mu$g/ml) and $\phi$ was also infected with $\phi$ and labeled with [H]uridine. Nucleotides were extracted from 100-$\mu$l samples at the indicated times with 4 M formic acid and resolved by thinlayer chromatography (7). Approximately 5% of the $\text{H}^1$H was in UTP and the remaining $\text{H}^1$H was in CTP. The specific activity of UTP (A) and CTP (B) was calculated from the $\text{H}^1$/[^3H] ratio for each nucleotide and the specific activity of [H]phosphate in the medium (7). (C) Incorporation of [H]uridine into RNA during each time interval was determined by precipitating 3-$\mu$l samples with cold trichloroacetic acid solution. (Greater than 95% of the trichloroacetic acid-insoluble $\text{H}^1$H was alkali labile.) 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 = [H]RNA/UTP + (y/x)CTP, where [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).

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

RNA from infected vegetative cells that hybridized to denatured $\phi$ DNA decreased from 22% (Fig. 2A) to 9.7% (Fig. 2B). Surprisingly, however, the proportion of $\phi$-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 $\phi$ 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 $\phi$-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 $\phi$ activity

The novel effect of chloramphenicol on $\phi$ transcription suggested that drug treatment of sporulating bacteria had relieved the inhibition of $\phi$ activity. To test this possibility RNA polymerase was purified partially by phase partitioning or ammonium sulfate fractionation from untreated and drug-
treated vegetative and sporulating cells and assayed with either φe 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 φe/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 φe after drug treatment (Table 2).

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 φe/poly(dA-dT) transcription ratios were computed from the linear portion of the activity curves. Chloramphenicol (CAM) and streptomycin (Str) treatments (150 μg/ml) were for 20 min before harvesting. Infected cells (multiplicity of infection = 5) were harvested 3–4 min after φe 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 φe/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 inset, Fig. 4A) while sporulation phase partitioned enzyme was deficient in σ polypeptide (gel inset, Fig. 4B). The gel inset 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.

### Table 2. Transcription ratios for RNA polymerase

<table>
<thead>
<tr>
<th>Purification</th>
<th>Drug</th>
<th>Phage infection</th>
<th>φe/poly(dA-dT)</th>
<th>Veg</th>
<th>T1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partitioned Enzyme</td>
<td>CAM</td>
<td>—</td>
<td>5.2</td>
<td>1.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Partitioned Enzyme</td>
<td>Str</td>
<td>—</td>
<td>5.0</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Enzyme</td>
<td>CAM</td>
<td>—</td>
<td>5.0</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Enzyme</td>
<td>φe</td>
<td>4.2</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Enzyme</td>
<td>CAM</td>
<td>φe</td>
<td>3.5</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 3. Time course of the effect of chloramphenicol on φe transcription and σ activity. (A) Vegetative and sporulating cells that had been treated with chloramphenicol (150 μg/ml) for various times were infected with φe 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 φe DNA. The percent input hybridizing at near saturating amounts of DNA is plotted for vegetative [3H]RNA (■) and sporulation [32P]RNA (○). (B) RNA polymerase was prepared by phase partitioning (3) from sporulating cells treated with chloramphenicol (150 μg/ml) for various times. The φe/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.
A inhibitor from E. coli is unstable in vivo (16).

Fig. 4. Transcription of ϕe DNA and poly(dA-dT) by RNA polymerase from vegetative, sporulating, and chloramphenicol-treated sporulating B. subtilis. Growth and sporulation was at 37°C 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 μg/ml) for 20 min. RNA polymerase was assayed (5) with [3H]ATP (8 Ci/mol) and 6 μg of ϕe DNA (●) or 10 μg of poly(dA-dT) (△) as templates. RNA polymerase was precipitated from 270 μg of each phase-partitioned enzyme with 100 μl 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 ϕe/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°C. 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°C (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 ϕe more actively in vitro 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 ϕe transcription in sporulating bacteria. As a further test of this idea, we have measured the rate of ϕe transcription in stationary phase cells of an asporogenous mutant, LS 3 (6, 20), that retains σ activity. We (manuscript in preparation) find that ϕe is transcribed about 2.5-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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