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Transcription After Bacteriophage SPP1 Infection in *Bacillus subtilis*

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The role of the host polymerase in *Bacillus subtilis* infected with phage SPP1 was studied in vivo with regard to production of phage-specific and host-specific ribonucleic acid (RNA) and to phage yield. Evidence is presented that the subunit(s) of *B. subtilis* RNA polymerase which is sensitive to rifampin and streptolydigin is necessary at all times during infection for phage production. The synthesis of phage RNA and the phage yield in strains resistant to either antibiotic were unaffected by the drug. Host RNA synthesis continued throughout infection; phage-specific RNA never accounted for more than 20% of pulse-labeled RNA at any time during infection.

A number of *Escherichia coli* bacteriophages have been thoroughly investigated with respect to transcription of their genomes during infection. Some of them (3, 4, 8) have been found to direct the synthesis of a new ribonucleic acid (RNA) polymerase, which is then responsible for transcription of a major portion of their deoxyribonucleic acid (DNA). Other phages are known to direct modifications of some of the subunits of the host-cell RNA polymerase (6, 16, 20) in order to complete transcription of their genome.

SPP1 is a virulent bacteriophage of *Bacillus subtilis* which has been isolated and characterized in this laboratory (14). Its small chromosome with easily separable strands and high infectivity of purified DNA have made it a useful tool for experiments on DNA replication (12), transcription (13), and recombination (7, 17, 18).

We report here results concerning the transcription process in *B. subtilis* infected with bacteriophage SPP1. These results show that at least a portion of the bacterial enzyme is necessary at all times after infection for phage production. Furthermore, possible modifications or substitutions of subunits in the host-cell RNA polymerase during infection do not alter the effect on the enzyme of rifampin and streptolydigin, two drugs that interact directly with RNA polymerase (2, 19). A similar investigation has been reported for *B. subtilis* infection with phage SPO1 (5).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. Spontaneous mutants resistant to rifampin or streptolydigin were selected by spreading about 10^9 bacteria on nutrient broth plates containing various concentrations of antibiotic. The strains chosen were all resistant to either antibiotic at a concentration of 100 μg/ml.

Media. M40 medium (11) supplemented with 0.5% glucose, 1% Norit-treated Casamino Acids (Difco), and 20 μg of tryptophan/ml was used for infection of liquid cultures. Adsorption medium was M40 without carbon sources (glucose, asparagine, Casamino Acids, and tryptophan). Plates of TY (15) agar and TY soft agar were used for standard plating techniques.

Chemicals. Sodium dodecyl sulfate (SDS) was from The British Drug Houses Ltd.; rifampin (molecular weight, 823) was a gift of G. Lancini, Lepetit Corp.; streptolydigin (molecular weight, 610) was a gift of G. B. Whitefield, The Upjohn Co.

Phage. SPP1 was purified from lysates either with a modification of the polyethylene glycol sodium dextran sulfate method (10) or, more recently, with a modification of the polyethylene glycol-NaCl method of Ymamoto et al. (21) and then by CsCl gradient centrifugation. Titration of phage was done by mixing 0.1 ml of the appropriate dilution in TY medium with 0.2 ml of *B. subtilis* PB 25 spores, leaving the mixture at 37 C for 30 min, and then adding 2.5 ml of soft TY agar at 45 C and plating on TY agar. More recently, we found no change in titers when the incubation step was omitted.

Infection. Liquid cultures were infected in the following way. An overnight culture in M40 medium was centrifuged and resuspended in adsorption medium at A_{540} = 1 and was shaken at 37 C for 60 min. At the end of this starvation period, RNA synthesis, as measured by short pulses of ^{14}C-uridine, was less than 0.5% of the initial synthesis immediately after resuspension of the cells in normal M40 medium. The cells were then infected with SPP1 at

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TABLE 1. *Bacillus subtilis strains used*

Strain	Genotype[a]			Origin
PB 25	*his-2*$^-$	*try-2*$^-$		M. Polsinelli
PB 3366	*his-2*$^-$	*try-2*$^-$	*stl*^{r}	From PB 25
PB 3367	*his-2*$^-$	*try-2*$^-$	*stl*^{r}	From PB 25
PB 3370	*his-2*$^-$	*try-2*$^-$	*rif*^{r}	From PB 25

[a] Symbols: *his*$^-$, *try*$^-$ = requirement for histidine and tryptophan; *stl*^{r}, *rif*^{r} = resistant to streptolydigin and rifampin, respectively.

a multiplicity of 10. After 5 min, the culture was rapidly chilled and centrifuged to eliminate unadsorbed phages and was then resuspended in complete M40 medium at 37 C. At this time, all cells behave as infected centers when plated with PB 25 spores as described above. A_{540} was usually measured at intervals, until lysis was complete.

Labeling RNA in infected cells. RNA synthesis was measured by 1- or 2-min pulses of ^{14}C-uridine (Amersham Radiochemical Center, 55 mCi/mmole). Usually, 0.9-ml portions of the culture were placed in tubes at 37 C containing about 1 μCi of uridine ($\sim$4.5 μg) and the tubes were shaken. The pulses were then terminated by adding 5 ml of 5% ice-cold trichloroacetic acid containing 100 μg of cold uridine/ml. The samples were left in ice for at least 1 hr and were then filtered on Whatman GF/C glass filters; the filters were washed with 20 ml of the same trichloroacetic acid solution, then with 15 ml of cold 0.01 M HCl, and finally with 95% ethanol. When RNA was to be extracted and purified, pulses (usually made on 25 ml of culture) were terminated by pouring the sample of the culture on 20 g of ice plus 50 mg of cold uridine and 2.5 mg of streptolydigin (Na salt), both dissolved in 1.5 ml of 1.3 M NaCl.

RNA extraction. The labeled cells were resuspended in ice-cold 0.1 M sodium acetate (*p*H 5.2)-0.01 M $MgCl_2$, layered on ice-cold water-saturated phenol, and treated at maximal output with an MSE ultrasonic disintegrator for a total of 3 min with interruptions every 30 sec, while cooling on ice. This homogenizes the mixture while disrupting the cells and, as judged by sedimentation profiles, is very effective in blocking nucleases. After sonic treatment, SDS was added to 1%, and three phenol extractions at 60 C were carried out (1). Finally, RNA was precipitated by adding one-third volume of 4 M NaCl and two volumes of ethanol and leaving the mixture at -20 C overnight. After centrifugation, the pellet was dissolved in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.5) and stored at -20 C. In the case of in vitro-synthesized RNA, the reaction mixtures were directly extracted at 60 C with phenol and SDS, and the same procedure was followed.

DNA extraction. DNA was extracted from CsCl-purified phage diluted in 0.01 M Tris-hydrochloride (*p*H 7.5), 0.01 M ethylenediaminetetraacetic acid (EDTA), and 0.05 M NaCl by three extractions with cold phenol saturated with 0.1 M Tris-hydrochloride (*p*H 7.5) and 0.01 M EDTA, and was dialyzed first against sterile 0.01 M Tris-hydrochloride (*p*H 7.5), 0.001 M EDTA, and 1.0 M NaCl and then against sterile 0.01 M Tris-hydrochloride, 0.001 M EDTA, and 0.1 M NaCl with repeated changes. Dialysis tubes were boiled for 60 min with 10% $NaHCO_3$, washed with distilled water, and stored in 50% ethanol-0.001 M EDTA.

Hybridization. Heat-denatured SPP1 DNA was hybridized to RNA in solution (0.4 ml), and hybrid formation was detected on nitrocellulose filters (Sartorius Membranfilter), according to the method of Nygaard and Hall (9). DNA excess conditions were chosen for this set of experiments and were checked for every RNA preparation.

RNA polymerase assay. RNA polymerase was extracted from *B. subtilis* PB 25 and its derivatives and was partially purified up to a complete DNA dependence by a modification of the procedure of Geiduschek and Sklar (5). In this procedure, cells were resuspended in 0.01 M Tris-hydrochloride (*p*H 7.9), 0.01 M $MgCl_2$, 0.005 M β-mercaptoethanol, and 0.001 M EDTA, sonically treated for 1 min with interruptions every 20 sec while cooling on ice, and centrifuged at 120,000 $\times$ *g* for 90 min. The supernatant fluid was brought to 65% saturation at 0 C with ammonium sulfate and was centrifuged at 13,000 $\times$ *g* for 30 min. The precipitate was resuspended in 0.01 M Tris-hydrochloride (*p*H 7.9), 0.01 M $MgCl_2$, 0.005 M EDTA, 10^{-4} M dithiothreitol, and 10% glycerol. The enzyme solution with an equal volume of 87% glycerol added can be stored at -20 C for at least 6 months without appreciable loss of activity. The assay mixture contained, in 0.20 ml, 0.1 M Tris-hydrochloride (*p*H 7.9) at 20 C, 0.01 M $MgCl_2$, 0.05 M KCl, 10^{-4} M EDTA, 2×10^{-4} M dithiothreitol, 10^{-3} M adenosine, guanosine, and cytidine triphosphate, 10^{-4} M ^{3}H-uridine triphosphate (20 μCi/μmole), and 0.1 mg of calf thymus DNA per ml. Assays were incubated for 10 min at 37 C, chilled in ice, and precipitated with 0.5 ml of 10% trichloroacetic acid containing 2% sodium pyrophosphate. After 5 min, the precipitate was collected on Whatman GF/C glass filters and washed twice with 10 ml of 2% trichloroacetic acid and then with ethanol. Radioactivity was determined by scintillation counting.

RESULTS

Phage RNA synthesis in infected cells. Figure 1 shows a typical SPP1 infection of *B. subtilis* strain PB 25. Cells from an overnight culture were resuspended in adsorption medium at a concentration of 10^8 to 3×10^8 cells/ml and infected at a multiplicity of infection of 10. After 5 min had been allowed for adsorption, the culture was cooled and rapidly centrifuged. The pelleted bacteria were then resuspended in complete medium which had been prewarmed at 37 C, and this was taken as time zero of infection. Phage production, under these conditions, reached a plateau after more than 1 hr after time zero. RNA synthesis, as measured by 2-min pulses of ^{14}C-

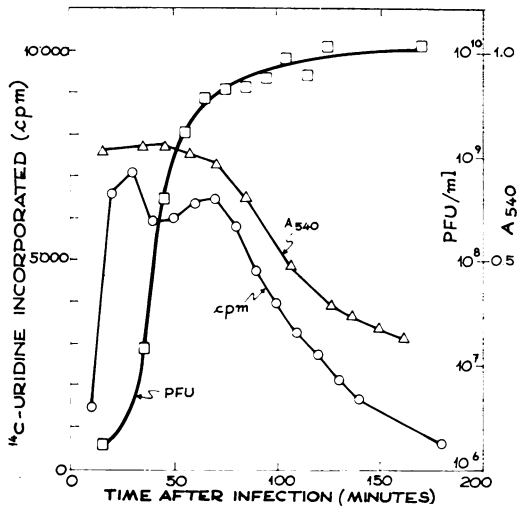

FIG. 1. *Infection of PB 25 with SPP1. An overnight culture of PB 25 in M40 supplemented with glucose, Casamino Acids, and tryptophan (see Materials and Methods) was centrifuged and resuspended in adsorption medium (carbon-free) to $A_{540} \sim 1$ (about 3×10^8 cells/ml). After 60 min, the cells were infected at a multiplicity of infection of 10. Time zero is the time at which the centrifuged infected cells were resuspended in complete M40 medium at 37 C. Symbols: △, A_{540}; ○, 2-min incorporation of ^{14}C-uridine (see Materials and Methods); □, intracellular phage (plaque-forming units/ml) as measured after adding saturating $CHCl_3$ to samples of the culture.*

uridine, continued throughout the infection period and paralleled, at late times, the absorbance curve.

The fraction of newly synthesized RNA which was phage-specific was rather low at all times of infection. Table 2 reports the percentage of pulse-labeled RNA that hybridized with denatured SPP1 DNA in the linear portion of a saturation curve (DNA excess). RNA was labeled for 3 min with ^{14}C-uridine after 20, 40, and 60 min of infection.

Table 2 also shows the hybridization efficiency of in vitro-synthesized SPP1 RNA. This was synthesized by use of *B. subtilis* PB 25 RNA polymerase and SPP1 native DNA as a template, and was highly asymmetric (*unpublished data*).

From a comparison of the hybridization efficiencies of the RNA from infected cells and of the RNA synthesized in vitro, a rough estimate can be made that no more than 20% of the pulse-labeled RNA after SPP1 infection is phage-specific. Therefore, even at late times after infection, a great proportion of the RNA synthesized is still host-specific. The results of hybridization to *B. subtilis* DNA are also given in Table 2. Although the hybridization efficiency in this case was much lower, the data suggest again that a large fraction of the RNA synthesized after infection is complementary to the host chromosome.

Effect of rifampin and streptolydigin on RNA synthesis and phage production. Addition of rifampin or streptolydigin to a growing culture of *B. subtilis* rapidly inhibited RNA synthesis (Fig. 2). Inhibition by streptolydigin was more prompt, as one should expect, considering that streptolydigin blocks both initiation and elongation of RNA chains at the same time (2) whereas rifampin inhibits initiation only.

The effect of addition of either antibiotic on phage production during SPP1 infection is shown in Fig. 3. At the indicated times, three different portions of the infected cultures were mixed with rifampin (20 μg/ml), streptolydigin (50 μg/ml), or $CHCl_3$. The portion to which $CHCl_3$ was added was immediately put on ice. The antibiotic-containing samples were further incubated at

TABLE 2. *Hybridization of pulse-labeled RNA from infected cells and of SPP1 RNA synthesized in vitro*[a]

RNA prepn	Percentage of input[b] hybridizing to SPP1 DNA	Percentage of input hybridizing to *B. subtilis* DNA
^{14}C-RNA from infected cells Labeling time		
From 20 to 23 min	8	—
From 40 to 43 min	8	5
From 60 to 63 min	10	—
^{14}C-RNA from uninfected cells (labeling time, 5 min)	<0.1	7
In vitro-synthesized RNA	48	—

[a] RNA from infected cells labeled with ^{14}C-uridine and purified as described in Materials and Methods was used. Hybridization was carried out in solution and was detected on nitrocellulose filters. The values reported are the averages of three points obtained in the linear portion of saturation curves (DNA excess). The SPP1 DNA concentration was 15 μg/ml in the experiments with RNA from infected cells and 20 μg/ml with in vitro-synthesized RNA. The *B. subtilis* DNA concentration was 45 μg/ml. Blank values (assays made in the absence of DNA) were always less than 0.2% of input radioactivity. In vitro RNA was synthesized in a mixture containing: 0.11 M Tris-hydrochloride (*p*H 7.5), 0.01 M $MgCl_2$, 0.05 M NaCl, 7×10^{-5} M EDTA, 2×10^{-4} M dithiothreitol, 10^{-3} M adenosine, guanosine, and uridine triphosphate, 2×10^{-4} M ^{14}C-cytidine triphosphate (5 μCi/μmole), 120 μg of SPP1 DNA/ml, and partially purified RNA polymerase from PB 25.

[b] Ribonuclease resistant.

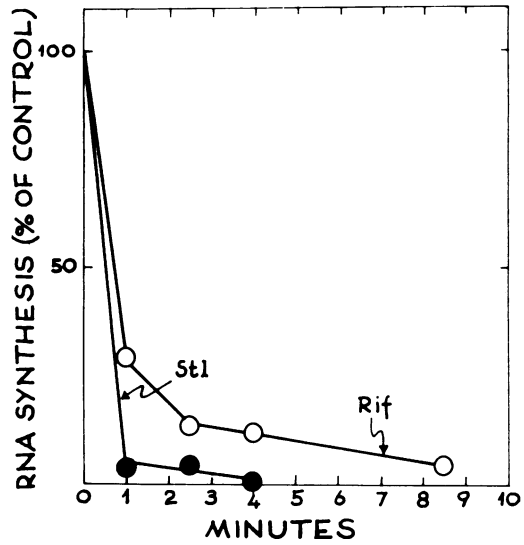

FIG. 2. *Inhibition of RNA synthesis by rifampin and streptolydigin. An overnight culture of PB 25 was centrifuged, resuspended in five times the original volume of fresh M40 medium, and shaken at 37 C for about 40 min. At this time, rifampin (50 μg/ml) or streptolydigin (50 μg/ml) was added (zero time in the figure). Pulses (1 min) were given to 0.9-ml portions of the culture (see Materials and Methods) and terminated by addition of cold 5% trichloroacetic acid containing 100 μg of cold uridine/ml. The results are expressed as percentages of a similar pulse given from 1.5 to 0.5 min before addition of the antibiotics. The time of the pulses, as plotted, represent the middles of the 1-min intervals. Symbols: ○, +50 μg of rifampin/ml; ●, +50 μg of streptolydigin/ml.*

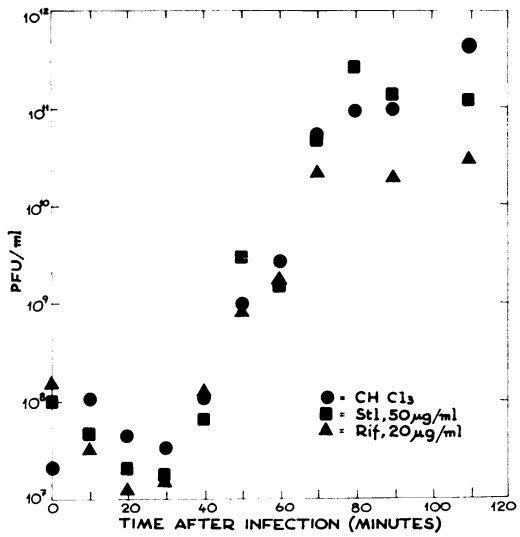

FIG. 3. *Effect of rifampin and streptolydigin on phage production. An overnight culture of PB 25 was centrifuged and infected (see Fig. 1) with a multiplicity of infection of 10. At the indicated times, samples were added to rifampin (20 μg/ml), streptolydigin (50 μg/ml), or $CHCl_3$ (see text). Incubation was continued until 160 min after infection, except for the $CHCl_3$-containing samples, which were immediately put on ice. At the end, $CHCl_3$ was added to all samples and the phages were titrated.*

37 C for 160 min, at which time $CHCl_3$ was added to all of them and to the original infected culture. The data show (within the experimental error) that at no time after infection can the number of bacteriophages, already present in the infected cells, be increased after addition of either antibiotic. In a similar experiment, the effect of streptolydigin on RNA synthesis was measured at various times after infection (Table 3). At the indicated times, two portions of the infected culture were transferred to two tubes, one containing streptolydigin to a final concentration of 40 μg/ml and the other a corresponding volume of buffer. At 3 and 6 min after the transfer, 1.5-min pulses of ^{14}C-uridine were given. Table 3 reports the per cent incorporation, measured 6 min after the addition of the antibiotic, relative to corresponding controls without antibiotic. As a comparison, the data for an uninfected culture are shown. It can be seen that the percentage of inhibition of RNA synthesis was of a similar magnitude in infected and uninfected cells. To assure that the low residual RNA syn-

TABLE 3. *Inhibition of RNA synthesis by streptolydigin during infection*[a]

Time of addition of streptolydigin (min)	Per cent incorporation
Infected cells	
15	3.1
30	5.6
45	6.9
60	4.3
75	4.1
90	4.6
105	5.6
120	7.7
Uninfected cells	
30	3.8
60	3.0

[a] The times indicated are those at which streptolydigin (40 μg/ml final concentration) in 0.1 M Tris-hydrochloride (pH 7.9) or a corresponding amount of Tris buffer were added to portions of the infected culture. At 6 min after the addition, 90-sec pulses of ^{14}C-uridine were given. The values reported represent per cent incorporation relative to that observed in the absence of streptolydigin. In the case of uninfected cells, the same procedure was followed as for infection (see Materials and Methods), except that no phage was added.

thesis still present after the addition of the antibiotic was not phage-specific, RNA labeled with ^{14}C-uridine from 70 to 73 min was extracted from infected cells to which streptolydigin had been added at 60 min after infection. The extracted RNA was then hybridized with an excess of SPP1 denatured DNA; only 10% of the input label was found in hybrid, showing no increase over the values reported in Table 2. Thus, the low residual DNA synthesis after addition of streptolydigin is not phage-specific.

From these results we can conclude that (i) there is no phage-specific RNA polymerase, synthesized after SPP1 infection, which is resistant to rifampin or streptolydigin and which can by itself support phage production, and (ii) transcription is necessary *at all times*, before the phage production reaches its maximal value, for the number of phages to increase.

Infection of PB 25 mutants resistant to rifampin and streptolydigin. We next looked for the possible synthesis of a phage-specific RNA polymerase sensitive to the two antibiotics. For this purpose, a number of *B. subtilis* mutants resistant to the two antibiotics were selected.

Table 4 shows the characterization of some resistant mutants selected from *B. subtilis* PB 25. RNA polymerase activity of partially purified extracts of these mutants was strongly resistant to either rifampin or streptolydigin. These mutants were then infected with SPP1 in the presence or absence of the antibiotic to which they were resistant. The lysis of the infected cultures was followed by monitoring absorbance at 540 nm. After lysis had occurred, $CHCl_3$ was added and the phage were titrated. From the results in Table 5, it can be seen that in none of the mutants analyzed was there any reduction of the phage yield in the presence of the antibiotic. In other words, no phage-specific RNA polymerase, sensitive to either rifampin or streptolydigin, and *necessary* for phage production, was synthesized after SPP1 infection.

TABLE 4. *RNA polymerase activity in partially purified extracts of PB 25 and resistant mutants*

Strain	Drug	Molarity in the assay	Percentage of control[a]
PB 25 *stl*^{s}, *rif*^{s}	Rifampin	0	100
		6×10^{-8}	22
		6×10^{-7}	6.5
		6×10^{-6}	0
		1.2×10^{-5}	0
PB 3370 *stl*^{s}, *rif*^{r}	Rifampin	0	100
		6×10^{-8}	100
		6×10^{-7}	100
		6×10^{-6}	100
		1.2×10^{-5}	100
PB 25 *stl*^{s}, *rif*^{s}	Streptolydigin	1.2×10^{-6}	100
		1.2×10^{-5}	38
		1.2×10^{-4}	0
		1.2×10^{-3}	0
PB 3366 *stl*^{r}, *rif*^{s}	Streptolydigin	1.2×10^{-6}	100
		1.2×10^{-5}	100
		1.2×10^{-4}	100
		1.2×10^{-3}	51
PB 3367 *stl*^{s}, *rif*^{s}	Streptolydigin	1.2×10^{-6}	100
		1.2×10^{-5}	100
		1.2×10^{-4}	100
		1.2×10^{-3}	53

[a] Values represent the percentage of controls without inhibitors. Control assays correspond to approximately 0.5 nmole of ^{3}H-uridine triphosphate incorporated into RNA in 10 min at 37 C.

TABLE 5. *Effect of rifampin and streptolydigin on phage production in strains sensitive or resistant to the inhibitors*[a]

Strain	Antibiotic added	PFU/ml	PFU (%)
PB 25 *stl*^{s}, *rif*^{s}	None	3.3×10^{11}	100
PB 25 *stl*^{s}, *rif*^{s}	Rif, 10 μg/ml	1.5×10^{8}	0.04
PB 25 *stl*^{s}, *rif*^{s}	Stl, 50 μg/ml	1.1×10^{8}	0.03
PB 3366 *stl*^{r}	None	5.0×10^{10}	100
PB 3366 *stl*^{r}	Stl, 40 μg/ml	5.0×10^{10}	100
PB 3367 *stl*^{r}	None	5.0×10^{10}	100
PB 3367 *stl*^{r}	Stl, 40 μg/ml	5.5×10^{10}	100
PB 3370 *rif*^{r}	None	6.3×10^{10}	100
PB 3370 *rif*^{r}	Rif, 50 μg/ml	7.5×10^{10}	100

[a] Rif = rifampin; Stl = streptolydigin; *stl*^{s}, *stl*^{r} = sensitive or resistant to streptolydigin; *rif*^{s}, *rif*^{r} = sensitive or resistant to rifampin; PFU = plaque-forming units.

DISCUSSION

Several conclusions can be drawn from our results. After infection of *B. subtilis* PB 25 with SPP1, no new RNA polymerase is synthesized which, alone, continues the infection process. More specifically, the two following possibilities can be excluded: (i) the synthesis of a new RNA polymerase, sensitive to rifampin and streptolydigin and necessary for phage production; and (ii) the synthesis at any time after infection of a new RNA polymerase resistant to either rifampin or streptolydigin and sufficient for phage production. Other possibilities cannot be excluded, but they seem rather unlikely. For ex-

ample, a new RNA polymerase could be made after infection, resistant to either rifampin or streptolydigin and necessary for infection, but such that it would be able to support phage production only in conjunction (at the same time) with the host enzyme.

On the basis of our results, modifications or substitutions of some of the subunits of the bacterial enzyme cannot be ruled out. It is important in this respect to observe that, if the subunit(s) responsible for the interaction with rifampin and streptolydigin is modified after infection, this does not alter its interaction with either antibiotic, both in the case of a sensitive and of a resistant strain. The most likely interpretation of our results is that the subunit(s) that confers resistance or sensitivity to the host RNA polymerase is not modified after infection. Its substitution with completely new, phage-specific subunits is subjected to the same criticism as the case discussed above (i) of a completely new enzyme.

We have established that host RNA synthesis continues throughout infection, but we do not know whether this is necessary for infection to proceed. What we do know is that RNA synthesis (host or phage or both) is always necessary for the phage yield to reach its maximum. Secondary effects of the antibiotics, distinct from inhibition of RNA synthesis (e.g., on assembly) are ruled out by the experiments with resistant mutants. This subject is currently under investigation.

From all of the experiments reported here, it is probably safe to conclude that no dramatic alteration of the transcription machinery occurs after SPP1 infection of *B. subtilis*. Most likely, the host RNA polymerase itself can completely transcribe DNA during infection. This hypothesis is also supported by some preliminary in vitro evidence.

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