RESISTANCE OF BACTERIOPHAGE PBS2 INFECTION TO RIFAMPICIN, AN INHIBITOR OF *Bacillus subtilis* RNA SYNTHESIS

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Received August 15,1972

Summary. The induction of viral enzymes, the synthesis of DNA, and the production of progeny phage during infection of *Bacillus subtilis* by bacteriophage PBS2 are all unaffected by the presence of rifampicin. Rifampicin, rifamycin SV, streptovaricin, and streptolydigin (all of which inhibit *B. subtilis* RNA synthesis by binding to DNA-dependent RNA polymerase) have no effect on PBS2 infection. However, actinomycin D and Lucanthone (which inhibit DNA-dependent RNA synthesis by binding to DNA) both prevent PBS2 replication. Therefore, PBS2 phage may utilize a rifampicin-resistant RNA polymerase to transcribe its uracil-containing DNA during phage infection.

Several antibiotics are known to be specific inhibitors of bacterial RNA biosynthesis (see reviews: Ref. 1 and 2). Some antibiotics directly inhibit DNA-dependent RNA polymerase *in vitro* and *in vivo*. For example, the rifamycins (including rifampicin) inhibit *Bacillus subtilis* RNA synthesis by binding to the enzyme to prevent it from initiating RNA chains (2, 3). The streptovaricins also inhibit initiation of RNA synthesis, probably by binding at the same site on RNA polymerase as rifampicin (4). Streptolydigin appears to inhibit *B. subtilis* RNA synthesis by blocking the elongation of RNA chains (5) by binding to RNA polymerase, probably at a site different from rifampicin (6). On the other hand, some antibiotics inhibit *B. subtilis* RNA synthesis by binding to the DNA template; examples are actinomycin D (1) and Lucanthone (7).

These antibiotics have been employed to demonstrate that RNA synthesis by host RNA polymerase is essential throughout infection of *Escherichia coli* by T4 phage (6,8,9) and λ phage (10) and throughout infection of *B. subtilis* by SPO1 phage (3) and β22 phage (11). Rifampicin also prevents MS2 phage development (12) and stops RNA synthesis (but not conversion of

single-stranded to double-stranded replicative DNA) during ϕX174 phage infection (13) in E. coli. While infection of E. coli by T7 phage (14) is sensitive to rifampicin during the first 3 minutes, rifampicin does not affect T7 replication later in infection, probably because a new phage-coded, template-specific RNA polymerase is induced which has been shown to be resistant to rifampicin (15).

Thus, all phage infections studied previously were sensitive to inhibition by rifampicin. We report here that infection of B. subtilis by the uracil-containing DNA phage PBS2 (16,17) is totally resistant to rifampicin. Similar observations were made independently by Rima and Takahashi.[1]

Materials and Methods. The methods for the growth of B. subtilis, infection by PBS2 phage, and preparation of extracts have been described (17). Antibiotics were from the following sources: rifampicin and rifamycin SV, Schwarz/Mann; actinomycin D, Sigma; Lucanthone (Miracil D), Calbiochem; and streptolydigin (U-5481) and streptovaricin (U-7750 complex), Dr. George Whitfield, Upjohn. Antibiotics were freshly dissolved in dimethylsulfoxide and added (as 1% of the culture volume) to cells. Control cultures received the same volume of dimethylsulfoxide (which had no detectable effect on PBS2 replication).

Results and Discussion. A typical time course of infection of B. subtilis by PBS2 phage is shown in Fig. 1. Approximately 90% of the cells are killed by phage and become infective centers; at least half of the "surviving cells" are phage-carrying pseudo-lysogens.[1] The phage adsorb and go through a 20 min eclipse period. The net synthesis of host DNA ceases at the time of infection, and DNA (phage DNA[1]) synthesis resumes after about 20 min. Host DNA remains stable, as judged by transformation, for 45 min (19). Progeny phage appear at 25 min; lysis begins at 35-40 min and proceeds slowly for 2 hr. We have found that addition of rifampicin (20 μg/ml) at 2 min before infection results in a DNA curve identical to that without rifampicin (Fig. 1). We have also observed superimposable curves (sampling every 15 min for 2 hr) for the culture absorbance at 660 nm and the total phage level for

[1] B. Rima and I. Takahashi, personal communication.

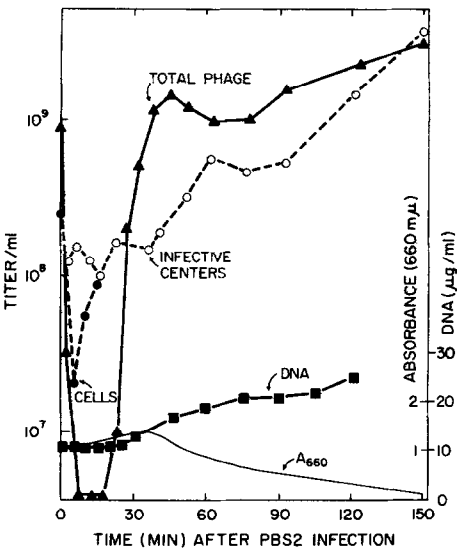

Fig. 1.

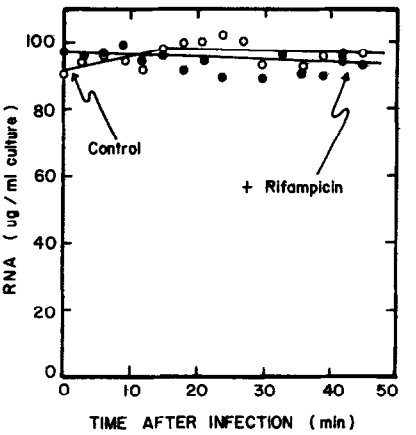

Fig. 2.

FIG 1. Time course of PBS2 phage infection of B. subtilis SB19. The infection was performed in Difco Penassay broth with vigorous shaking at 37° using a multiplicity of 5 phage per cell. Cells and phage were titered as described previously (17). Total phage were determined by plating cultures prematurely-lysed with KCN and lysozyme (3). The culture absorbance at 660 nm (A660) in a 1 cm cuvette was monitored at 5 min intervals. Using the methods of Warner and Barnes (18), cultures were treated with trichloroacetic acid and assayed by the indole-HCl method for DNA, using salmon sperm DNA (Calbiochem) as a standard.

FIG 2. RNA concentration in cultures of PBS2-infected B. subtilis SB19 in the presence and absence of rifampicin. A culture grown to A_{660} = 1.0 as in Fig 1 was infected (o) with PBS2 phage at a multiplicity of 15, or treated with rifampicin (•) at 20 μg/ml for 2 min before infection. Aliquots were treated as in Fig. 1 for assay by the orcinol-HCl method for RNA (20) using yeast RNA as a standard.

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cultures treated with 0, 20, or 100 μg of rifampicin per ml at 5 min before infection. Thus, rifampicin had no detectable effect on these parameters of PBS2 infection.

However, the B. subtilis cells are sensitive to rifampicin. No colonies (<0.1%) will grow on plates containing rifampicin at 1 μg/ml. Pulse-labelling of RNA is effectively inhibited (see below) by rifampicin. For wild-type B. subtilis, 1 μg rifampicin per ml eliminates RNA synthesis in vivo and in vitro (3). Thus, PBS2 infection appears not to require the

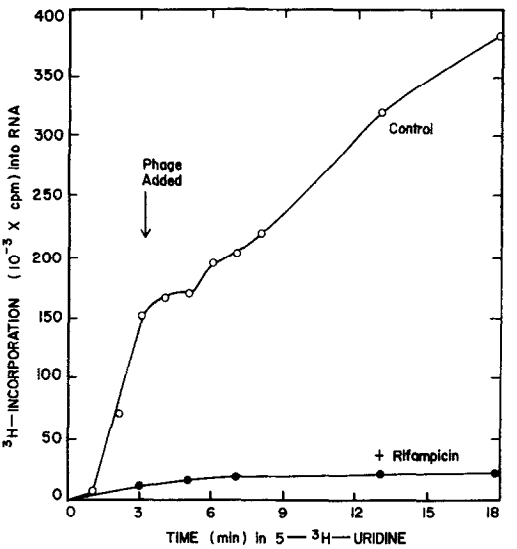

FIG 3. Incorporation of 5-[^{3}H]-labelled uridine into RNA by uninfected and PBS2-infected B. subtilis SB19 in the presence and absence of rifampicin. To a culture grown as in Fig. 1 was added tritiated-uridine (o) (final concentration of 50 nmole/ml at a specific activity of 16,000 cpm/nmole), or 100 µg rifampicin per ml (●) at 3 min before the tritiated-uridine. PBS2 phage at a multiplicity of 10 (in 5% of the culture volume) was added 3 min after the tritiated-uridine to both cultures. Continuous labelling of RNA was monitored by removing 1 ml aliquots to 1 ml ice-cold 10% trichloroacetic acid, collecting the precipitate on Whatman GF/A discs, drying at 120°, and counting radioactivity as described earlier (17). The amount of this incorporation actually in DNA (acid-precipitable material resistant to incubation in 0.5 N KOH for 20 hr at 30°) was low and essentially constant during this period of infection (8,000 or 2,000 cpm for cultures in the absence or presence of rifampicin, respectively).

activity of the host's RNA-synthesizing system, which is sensitive to rifampicin. In fact, PBS2 infection appears to result in the cessation of net RNA synthesis after 10-15 min (Fig. 2), in agreement with the results of Paleček (19). Addition of rifampicin (20 µg/ml) at 3 min before infection prevents all colorimetrically-detectable RNA synthesis (Fig. 2) without affecting PBS2 replication. The nature of the residual RNA synthesis occurring after normal PBS2 infection is under investigation.

In cultures continuously labelled by 5-[^{3}H]-uridine (Fig. 3), there is a sudden 80% decrease in the apparent rate of RNA synthesis after PBS2 infection, reflecting the shut-off of colorimetrically-detectable

RNA synthesis seen in Fig. 1. When rifampicin (100 μg/ml) was added, only a small amount of [^{3}H]-uridine incorporation was observed in infected cells (less than 10% of the rate without rifampicin).

In cultures pulse-labelled by [^{3}H]-uridine for 3 min (data not shown), a large decrease (75-90%) in incorporation was observed immediately after infection by PBS2 phage. When rifampicin (20 μg/ml) was added 3 min before the [^{3}H]-pulse, incorporation into uninfected cells was inhibited by 97%; however, infected cells showed a continuous increase in [^{3}H]-incorporation after rifampicin treatment, from 5 to 45 min after infection. The final rate (for a 3 min pulse at 45 min) was 10% of the value for an untreated, uninfected culture (although one-half of the radioactivity incorporated in infected cells was actually in DNA). Thus, PBS2 infection results in the appearance of RNA whose synthesis is insensitive to inhibition by rifampicin. Rima and Takahashi have demonstrated that this rifampicin-resistant RNA hybridizes specifically to phage DNA.[1]

Finally, as a more sensitive test of a possible effect of rifampicin on the timing of PBS2 transcription, we assayed for the production of PBS2-induced enzymes. As shown in Table I, the specific activity of 2 phage enzymes and the phage yield were similar in the presence and absence of rifampicin (and were independent of the level or the time of addition of rifampicin). In other experiments (data not shown) using rifampicin (100 μg/ml) at 5 min before infection, the time course of induction paralleled precisely the normal induction of PBS2 deoxythymidylate-deoxyuridylate phosphohydrolase[2] and PBS2 DNA polymerase (17). Thus, rifampicin does not influence PBS2 enzyme induction. Whether the synthesis of host enzymes is prevented after PBS2 infection is under investigation.

Other known specific inhibitors of RNA synthesis were tested for their effect on PBS2 infection. RNA synthesis by uninfected cells was strongly inhibited by the 6 antibiotics tested at 20 μg/ml (Table II). Total

[2] A. Price and S. Fogt, manuscript in preparation.

TABLE I. Relative phage production and enzyme induction during PBS2 infections in the presence and absence of rifampicin.

Experiment	Strain	Rifampicin Level	Rifampicin Time	Phage yield	DNA polymerase	dTMPase-dUMPase
		µg/ml	min			
I	SB19	5	+5			0.7
II	SB19	20	+1/2	0.9	1.0	1.0
III	1306	20	+1/2	1.3	0.9	0.9
IV	SB19	100	-5	1.4		

Infections were performed at 37° using strain SB19 (wild-type) or strain 1306 (*pol*⁻, *met*⁻, *leu*⁻ from Julian Gross) with a multiplicity of 5 phage per cell. Rifampicin was added at the indicated concentration and time after infection. Phage yield was determined at 2 hr after infection. Extracts were prepared after 45 min infection and were assayed for DNA polymerase (17) and dTMPase-dUMPase (see footnote 2) specific activity. All the values presented have been normalized to the control for each experiment without rifampicin (typical values were 4×10^9 phage per ml, 150 pmoles/min/mg protein of dATP incorporated, and 0.2 µmole/min/mg protein of phosphate released, respectively).

phage titers showed an eclipse at 10 min (Fig. 1) for all infections, but only Lucanthone and actinomycin D prevented progeny phage production (Table II). Actinomycin D also prevents PBS2 dTMPase[2] and DNA polymerase (17) induction. Lucanthone and actinomycin D interact directly with DNA to prevent transcription (1, 7), suggesting that RNA synthesis is essential for PBS2 replication.

However, the antibiotics (rifampicin, rifamycin SV, streptovaricin, and streptolydigin) which directly interact with *B. subtilis* RNA polymerase to prevent transcription (1, 2, 4, 5) had no significant effect on PBS2 phage production (Table II). This may indicate that PBS2 infection does not require the activity of the host's RNA polymerase [or at least its subunit(s) sensitive to these antibiotics] and that PBS2 DNA transcription utilizes an RNA polymerase insensitive to these antibiotics. Since PBS2 infection

TABLE II. Relative effect of various antibiotics on RNA synthesis and PBS2 replication in B. subtilis SB19

Antibiotic	Uridine incorporation	Phage yield
	%	%
None	100	100
Rifampicin	5	70
Rifamycin SV	6	95
Streptovaricin	11	70
Streptolydigin	10	75
Lucanthone	25	15
Actinomycin D	7	<10

Antibiotics at a concentration of 20 μg/ml were added to uninfected cells 5 min before a 3 min-pulse in tritiated-uridine (as in Fig. 3), or 5 min before phage infection at a multiplicity of 10. Values are expressed as a percentage of the control values without antibiotic (135,000 cpm/ml [^{3}H]-uridine incorporated in 3 min, and 3 x 10^9 phage per ml after 2 hr infection).

is totally resistant to high concentrations of these antibiotics added even before the phage, perhaps the PBS2 virion contains an RNA polymerase [as recently discovered (21) in Pseudomonas phage PM2] which is insensitive to these antibiotics and which is responsible for transcription of the uracil-containing PBS2 DNA.

Initial experiments using B. subtilis RNA polymerase prepared as described by Losick and Sonenshein (22) indicate that poly(dA-dT) is an excellent template, while PBS2 DNA is a very poor template. Losick and Sonenshein (22) made similar observations using DNA from other sources. Thus, we have been unable to eliminate the possibility that transcription of PBS2 DNA by B. subtilis RNA polymerase is insensitive to rifampicin. Experiments to characterize RNA polymerase activities in uninfected and infected cells and possibly in PBS2 virions are in progress.

Acknowledgments. We thank Anne Cahill and Suzanne Fogt for performing some of the experiments. Support for M. F. was from N.I.H. Training Grant GM00187. This work was supported by the U.S. Atomic Energy Commission (Report No. COO-2101-7) and by the Horace H. Rackham School of Graduate Studies (FRF-124).

Brief presentations of this research were made at the Annual Meeting of the American Society for Microbiology (Philadelphia, April, 1972) and the Bacteriophage Meetings (Cold Spring Harbor, August, 1972).

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