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Rifampicin-resistant bacteriophage PBS2 infection and RNA polymerase in Bacillus subtilis

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ABSTRACT

Bacteriophage PBS2 replication is unaffected by rifampicin and other rifamycin derivatives, which are potent inhibitors of *Bacillus subtilis* RNA synthesis. Extracts of gently-lysed infected cells contain a DNA-dependent RNA polymerase activity which is specific for uracil-containing PBS2 DNA. The PBS2-induced RNA polymerase is insensitive to rifamycin derivatives which inhibit the host's RNA polymerase.

INTRODUCTION

Price and Frabotta[1] and Rima and Takahashi[2] have previously shown that viral enzyme induction, DNA synthesis, and progeny phage production during PBS2 phage infection of *Bacillus subtilis* are all unaffected by the presence of rifampicin, rifamycin SV, streptovaricin, and streptolydigin (all known to bind to and inhibit the *B. subtilis* RNA polymerase), even when these drugs were added to cells prior to infection. However, viral mRNA synthesis and expression were prevented[1,2] by the addition of actinomycin D (known to inhibit RNA synthesis by binding to the DNA template). Thus, it was proposed[1,2] that PBS2-infected cells contain a rifampicin-resistant, actinomycin-sensitive RNA polymerase which differs from the known rifampicin-sensitive host RNA polymerase[3-5].

Other prokaryotic RNA synthetic enzymes have been described which are naturally insensitive to rifampicin. They include an *Escherichia coli* poly A polymerase[6], a poly G polymerase induced by *E. coli* satellite phage P4[7], a *Pseudomonas* gh-1 phage-induced RNA polymerase[8], and the *E. coli* T3 and T7 phage-induced RNA polymerases[9]. However, PBS2 phage appears to be unique in that the whole *in vivo* process of RNA synthesis and phage replication is rifampicin-resistant [although one analogous system may be the recently described rifampicin-resistant mRNA synthesis by *E. coli* phage N4[10]]. We report here that PBS2 infection of *B. subtilis* results in the induction of a PBS2 DNA-dependent RNA polymerase activity which is insensitive *in vitro* and *in vivo* to several rifamycin derivatives.

MATERIALS

In addition to the antibiotics obtained previously, the rifamycin AF compounds were the gift of Drs. G. Lancini and L. Silvestri of Gruppo Lepetit. Native *B. subtilis* and salmon sperm DNA were purchased from Calbiochem and poly dAT from Sigma. The [8-^{3}H]ATP and [5-^{3}H]UTP were from ICN Pharmaceuticals.

RESULTS AND DISCUSSION

The data in Table I confirm our previous results[1] that PBS2 phage production is insensitive to prior addition of rifampicin but is totally blocked by actinomycin D. In addition we show here that 8 newer rifamycin AF derivatives[11,12] have only slight inhibitory effects on PBS2 replication. In contrast, all of these drugs are potent inhibitors of *B. subtilis* RNA synthesis (Table I), mainly by inhibiting the cell's RNA polymerase[11] (but see discussion below). Thus PBS2 mRNA appears to be synthesized by a rifampicin-insensitive RNA polymerase.

Table II indicates that PBS2-infected cells do indeed contain a rifampicin-resistant RNA polymerase activity. The extract was prepared essentially DNA-free by gentle freeze-thaw lysis, leaving the RNA polymerase activity

TABLE I. Influence of certain drugs on RNA synthesis and PBS2 replication in *B. subtilis* SB19

Drug	Concentration	RNA Synthesis	Phage Yield
	µg/ml	%	%
None added	0	100	100
Rifampicin	50	1.1	90
Rifamycin AF/05	50	0.1	40
" BO	50	0.5	50
" DNFI	50	2.0	50
" 01	50	0.1	60
" ABDP-cis	50	0.8	40
" 013	50	0.1	40
" AP	50	0.8	70
" 015	50	0.1	70
Actinomycin D	10	0.7	1

Drugs at the indicated final concentrations were added in 50 µl of dimethylsulfoxide to 1 ml of uninfected cells 5 min before a 3 min pulse (see Ref. 1) in [5-^{3}H] uridine (final concentration of 50 nmole/ml at a specific activity of 16,000 cpm/nmol), or 5 min before PBS2 phage infection at a multiplicity of 10. Values are expressed as a percentage of the control values without drug (56,000 cpm/ml uridine incorporated during RNA synthesis, and 5.6 x 10^9 phage/ml after 135 min infection).

TABLE II. PBS2 RNA polymerase activity in a gently-lysed infected cell extract: dependence on added DNA and effects of antibiotics

DNA added		Antibiotic added	[^{3}H]UTP incorporated
	µg/ml		cpm
PBS2 phage	26	None	7770
PBS2 phage	26	rifampicin	7990
PBS2 phage	26	actinomycin D	0
None	0	rifampicin	350
B. subtilis	20	rifampicin	110
salmon sperm	20	rifampicin	50
poly dAT	26	rifampicin	90

B. subtilis SB19 was grown in 500 ml Penassay broth at 37°, infected with 5 PBS2 phage per cell for 35 min, and harvested as described previously (Ref.13). Infected cells were washed at 4° in 25 ml of 10 mM TrisHCl buffer, pH 7.6, containing 1 mM EDTA. The pelleted cells were resuspended in 5 ml of 20 mM TrisHCl, pH 8.5, containing 200 mM NaCl, 2 mM EDTA, 10 mM mercaptoethanol, and 1 mM phenylmethyl-sulfonylfluoride. The suspension was frozen for 8 h, quickly thawed and mixed well at 37°, and allowed to lyse undisturbed at 4° for 12 h. Five hours after careful addition of 2.5 ml of 5 M NaCl in the above buffer, the clarified lysate was centrifuged at 39,000 x g for 2 h to separate the clear supernatant extract from the viscous pellet (containing membrane-bound DNA, unlysed cells, and debris). The extract was dialyzed in 50 mM TrisHCl, pH 7.5, containing 10 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.02% NaN_3 as preservative. The 7.3 ml of dialyzed extract contains 3.5 mg protein/ml (60% of the total soluble protein) and 18 µmol/h/mg protein of PBS2-induced dTMP phosphohydrolase activity (80% of total) measured as described previously (Ref. 13).

PBS2 DNA was isolated as follows: phage were precipitated from a 10 l lysate by 6.5% polyethylene glycol in 0.3 M NaCl (Ref. 14). After resuspension in 100 ml absorption medium (Ref. 15) and 3 clarifying centrifugations at 5,000 x g for 15 min, the concentrated phage suspension was layered in 4 tubes each containing 7 ml CsCl (1.3 g/cc) over 7 ml CsCl (1.5 g/cc). After centrifugation for 3 h at 20,000 rpm in a SW27 rotor at 4°, the phage were collected from the interface of the 2 CsCl sulutions, diluted to 80 ml, pelleted by centrifugation for 2 h at 20,000 x g, and resuspended in 10 ml citrate buffer (Ref. 15). Phage were disrupted by agitation with 0.2 g sodium dodecyl sulfate for 5 min, followed by 3 extractions with 10 ml aqueous phenol and then ether. The ether-free DNA solutions were dialyzed twice in 500 ml citrate buffer.

Antibiotics were added in 2 µl of dimethylsulfoxide to give a final drug concentration of 40 µg/ml in the reaction mixture (250 µl).

RNA polymerase activity was measured under conditions identical to those of Losick and Sonenschein (Ref. 4) except that KCl was omitted. Reaction mixtures contained 7.5 µC$_i$ [^{3}H]UTP (0.15 mM) with 25 µl of extract for a 15 min incubation at 37°. Tritium was counted on membrane filters at an efficiency of about 25%, and zero-time control values of 640 cpm have been subtracted from the data presented. Under these conditions, the amount of incorporation is directly proportional to the amount of extract added and the time of incubation.

dependent upon added DNA. This activity required the presence of all four ribonucleoside triphosphates in the assay. It was unaffected by rifampicin, blocked by actinomycin D, and appears to be specific for PBS2 DNA (Table II). The other DNA species employed were tested in control incubations using purified *E. coli* K12 RNA polymerase (2 units, Sigma); these gave incorporations of about 6,000 cpm for PBS2, *B. subtilis*, and a salmon sperm DNA and 19,000 cpm for poly dAT under the above conditions, and all were totally inhibited by rifampicin. PBS2 DNA is known to be essentially inactive as a template for the *B. subtilis* RNA polymerase (Ref. 2,5 and J. Pero and R. Losick, *personal communication*), perhaps suggesting a physiological requirement for a new PBS2 RNA polymerase.

The data in Table III show that little or no rifampicin-resistant RNA polymerase activity was present in uninfected cell extracts. The induction of this activity appears to begin after 5 min and increases during infection to its highest levels near the end (35 min; see Ref. 1) of infection. Appearance of the polymerase was prevented by addition of chloramphenicol or actinomycin D at the time of infection (Table III), but induction was not affected

TABLE III. Induction of PBS2 RNA polymerase activity

Extract of treated cells	[^{3}H]ATP incorporated
	cpm
Uninfected	<100
35 min infected + chloramphenicol	<100
35 min infected + actinomycin D	<100
5 min infected	170
20 min infected	2500
35 min infected	5150

B. subtilis 1306 pol$^-$ was grown to 2 x 10^8 cells/ml, and rifampicin was added to 100 µg/ml. Aliquots were either left shaking at 37^o uninfected for 15 min, or infected with PBS2 phage for the indicated times, alone or in the presence of chloramphenicol (100 µg/ml) or actinomycin D (10 µg/ml). Cell extracts were prepared (2.3 to 2.7 mg protein/ml) as in Table II and dialyzed in buffer containing 0.1 mM phenylmethyl-sulfonylfluoride without NaN_3. Assays for RNA polymerase activity were performed as in Table II, except that 4.2 μC_i [^{3}H]ATP (0.15 mM) was used; control values for reactions lacking DNA were subtracted. Incubations were for 15 min in the presence of PBS2 DNA (26 µg/ml), rifampicin (40 µg/ml), and 25 µl of extract.

by the presence of rifampicin (see legend to Table III). Thus, induction of this enzyme is similar to that of other PBS2 enzymes: dCTP deaminase[16], DNA polymerase[15], and dTMP phosphohydrolase[13]. We previously[1] suggested the possibility that the PBS2 virion contained a rifampicin-resistant RNA polymerase which was injected into the cell during infection. The significance of our failure to detect activity in infected cells pretreated with chloramphenicol is not clear, since we do not know the minimum number of injected RNA polymerase molecules which our assay could detect. Similarly, we have been unable to detect activity in PBS2 phage particles or phage heads purified in CsCl density gradients.

The results in Table IV indicate that the PBS2-induced RNA polymerase activity is relatively resistant to several rifamycin analogues, streptolydigin, and streptovaricin. Several of these antibiotics bind to the β-subunit of the *B. subtilis* RNA polymerase[11] and to other phage RNA polymerases[17], although some bind non-specifically to many proteins[12]. Yet the PBS2 enzyme is sensitive to lucanthone and actinomycin D, which inhibit RNA synthesis by binding to the DNA template. Thus, the effects of these drugs on the PBS2 RNA polymerase activity (Table IV) and on PBS2 phage reproduction *in vivo* (Table I and Ref. 1) are qualitatively similar.

TABLE IV. Sensitivity of PBS2 RNA polymerase activity to various drugs

Drug	[^{3}H]ATP incorporated
	cpm
None added	3000
Rifampicin	2800
Rifamycin AF/05	2400
" BO	3000
" DNFI	1800
" 01	2300
" ABDP-cis	3000
" 013	2100
" AP	2800
" 015	2000
Streptolydigin	3000
Streptovaricin	2900
Lucanthone	700
Actinomycin D	200

Extract aliquots (25 µl containing 58 µg protein) of 35 min-infected *B. subtilis* 1306 pol$^-$ cells were assayed in the presence of each drug at 40 µg/ml as described in Table III.

Independent results on the detection and purification of the PBS2 RNA polymerase have been obtained by Clark et al.[18]. It remains to be determined whether the PBS2 virion contains and injects all or part of this enzyme or its mRNA into the cell during infection, or whether some as yet uncharacterized host enzyme catalyzes the rifampicin-resistant synthesis of mRNA coding for the PBS2 RNA polymerase.

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REFERENCES

1 Price, A. R., and Frabotta, M. (1972) Biochem. Biophys. Res. Commun. 48, 1578-1585
2 Rima, B. K., and Takahashi, I. (1973) Canad. J. Biochem. 51, 1219-1224
3 Avila, J., Hermoso, J. M., Vinuela, E., and Salas, M. (1971) Eur. J. Biochem. 21, 526-535
4 Losick, R., and Sonenshein, A. L. (1969) Nature 224, 35-37
5 Maia, C. C., Kerjan, P., and Szulmajster, J. (1971) Fed. Europ. Bioc. Soc. Lett. 13, 269-274
6 Ohasa, S., and Tsugita, A. (1972) Nature New Biol. 240, 35-41
7 Barrett, K. J., Gibbs, W., and Calendar, R. (1972) Proc. Nat. Acad. Sci. USA 69, 2986-2990
8 Towle, H. C., Jolly, J. F., and Boezi, J. A. (1973) Federation Proc. 32 645
9 Dunn, T. T., Bautz, F. A., and Bautz, E. K. F. (1971) Nature New Biol. 230, 94-96
10 Rothman-Ames, L. B., Hill, J., and Schito, G. C. (1974) Abst. Ann. Meet. Amer. Soc. Microbiol. p. 228
11 Riva, S., and Silvestri, L. G. (1972) Ann. Rev. Microbiol. 26, 199-224
12 Riva, S., Fietta, A., and Silvestri, L. G. (1972) Biochem. Biophys. Res. Commun. 49, 1263-1271
13 Price, A. R., and Fogt, S. M. (1973) J. Biol. Chem. 248, 1372-1380
14 Yamamoto, K. R., Alberts, B. M., Benzinger, R., Hawthorne, L., and Treiber, G. (1970) Virology 40, 734-744
15 Price, A. R., and Cook, S. J. (1972) J. Virol. 9, 602-610
16 Tomita, F., and Takahashi, I. (1969) Biochim. Biophys. Acta 179, 18-27
17 Chamberlain, M. J., and Ring, J. (1972) Biochem. Biophys. Res. Commun. 49, 1129-1136
18 Clark, S., Losick, R., and Pero, J. (1974) Nature, in press