Abortive Infection of *Bacillus subtilis* Bacteriophage PBS1 in the Presence of Actinomycin D

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Received for publication 23 January 1969

Actinomycin D caused the irreversible loss of PBS1 phage infectious centers and PBS1-mediated transductants. The loss of infectious centers occurred only within the first 4 min after the addition of phage to cells. Actinomycin did not inactivate free phage or inhibit phage adsorption. Electron micrographs indicated that phage adsorbed to cells in the presence of actinomycin ejected their deoxyribonucleic acid (DNA) normally. However, when cells were infected in the presence of actinomycin, 15 to 22% of their ³²P-labeled DNA appeared in the medium, whereas only 1.5 to 7.2% of the ³²P-labeled DNA appeared in the medium during normal infection. Neither 8-azaguanine nor chloramphenicol caused a similar loss of PBS1 infectious centers or transductants. Actinomycin also caused the loss of SP10 infectious centers but it had no effect on SP01 or ϕ 29 infections. We conclude that actinomycin causes abortion of PBS1 infection by inhibiting the uptake or retention of phage DNA into host cells. The immunity of SP01 and ϕ 29 infections to actinomycin probably reflects differences in the penetration mechanisms of these phages.

Abortion of T2 infection occurs when the enzymatic activity of the phage-host complex is inhibited by low temperature (1). Presumably the loss of infectious centers which occurs in the first 2 min of infection is due to the "splitting" or ejection of phage deoxyribonucleic acid (DNA) from the phage protein without its subsequent uptake into the inhibited host cell (7). A dependence upon the host cell metabolism for successful penetration is common to a number of phage-host systems. Anaerobiosis prevents the transfer to host cells of 90% of T5 phage DNA (3), and cyanide prevents the completion of T4 phage DNA transfer to host cells (H. Reiter, Ph.D. Thesis, The George Washington Univ., Washington, D.C., 1965). Although cyanide inhibition is largely reversible, an increasing number of infectious centers are aborted as the period of cyanide inhibition is lengthened.

The penetration of host cells by the flagella-specific *Bacillus subtilis* phage PBS1 is also reversibly inhibited by cyanide (8). In this case, cyanide blocks a host-mediated step leading to the irreversible adsorption of the PBS1 phage. Phage adsorbed in cyanide are completely recovered as infectious units. In this paper, we report the abortive infection of *B. subtilis* by PBS1 and

SP10 phages, and the successful infection of *B. subtilis* by ϕ 29 and SP01 phages in the presence of actinomycin D. We show that abortion occurs within the first 4 min of infection, and we present evidence indicating that phage DNA is ejected but that DNA uptake into the cell is incomplete in the presence of actinomycin D.

MATERIALS AND METHODS

Media. The enriched, tryptone-yeast extract broth (ETY), the minimal medium, and the phosphate buffer used for phage suspension and dilution were described by Pritikin and Romig (6). The EMTA medium used to label PBS1 phage DNA with ³²P contained 3,6-endomethylene-1,2,3,6 tetrahydrophthalic acid, 0.9 g; NaCl, 0.29 g; (NH₄)₂SO₄, 0.2 g; L-glutamic acid, 0.1 g; sodium citrate, 0.085 g; MgSO₄·7H₂O, 0.02 g; CaCl₂, 0.01 g; KH₂PO₄, 0.006 g; K₂HPO₄, 0.014 g; MnCl₂, 0.126 mg; FeCl₃·6H₂O, 0.025 mg; glucose, 0.5 g; glass-distilled water, 100 ml. The medium was neutralized before autoclaving by adding 0.5 ml of 45% KOH and then adding enough NaOH to bring the pH to 7.1 to 7.2.

Organisms. *B. subtilis* strains 168, SB19, and SB171 were used in these studies. SB19 is a streptomycin-resistant, PBS1-sensitive prototroph derived from *B. subtilis* 168. SB171 is a nonflagellated derivative of 168. It does not adsorb PBS1 phage. Phages PBS1, SP10, SP01, and ϕ 29 have been described by Takahashi (10), Thorne (11), Okubo, Strauss, and Stodolsky (5), and Reilly and Spizizen (9), respectively.

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Preparation of PBS1 and ^{32}P -labeled PBS1 phage. Approximately 2×10^7 SB19 cells, in EMTA medium, were infected with PBS1 phage at a plaque-forming unit (PFU) to bacterium ratio (PBR) of 1.5. The total volume of this infection medium was 0.2 ml. After a 15-min adsorption period at 37 C, in a stationary tube, 3 ml of EMTA medium containing $6.4 \mu\text{C}$ of $\text{H}_3^{32}\text{PO}_4$ /ml was added, and the culture was aerated at 37 C for another 6 hr. The culture was then added to 100 ml of EMTA medium containing $6.4 \mu\text{C}$ of ^{32}P /ml. This was in a 1-liter Erlenmeyer flask which was then incubated at 37 C on a reciprocating shaker for 16 hr. After this the culture was stored at 4 C for 20 hr. Bacterial cells and other debris were then removed by centrifugation at $6,000 \times g$ for 10 min. The ^{32}P -labeled phage in this cleared lysate was purified by centrifugation in a CsCl density gradient. CsCl was added directly to the lysate, 6.2 g of CsCl per 10 ml of lysate. This mixture was centrifuged for 24 hr at 30,000 rev/min in a Spinco SW-41 rotor. The visible band near the center of each tube was collected and dialyzed against 1,000 volumes of phosphate buffer. CsCl was added to the dialysate, and the phages were again banded, collected, and dialyzed as above.

Unlabeled phage were prepared in the same way except that only one CsCl centrifugation was used. Unlabeled phage were also prepared by centrifuging the phage from cleared lysates at $47,120 \times g$ for 60 min, resuspending the pelleted phage in phosphate buffer, and preceding through the CsCl centrifugation and dialysis procedures as described above.

Assay of PBS1. The agar overlay technique was used to assay PBS1 by plaque count. Diluted phage (0.1 ml) was mixed with 2×10^7 to 2.5×10^7 *B. subtilis* SB19. After 10 min at 37 C to allow for phage adsorption, 2.5 ml of molten (50 C) 0.5% agar-ETY was added, and this mixture was poured as a uniform layer on the surface of a 1.5% agar-ETY plate. When infected bacteria were titrated by plaque assay, the adsorption period was omitted and the infected cells were diluted in chilled media to prevent premature cell lysis.

Electron microscopy. Cells were grown in ETY broth to a concentration of about 7.6×10^8 cells/ml. PBS1 phage was added to a final concentration of 2.1×10^9 PFU/ml (PBR of about 2.8). After 10 min of vigorous aeration at 37 C, the culture was fixed with 0.5% Formalin. The agar filtration technique developed by Chuen-Mo To (12) was used to prepare the fixed culture for electron microscopy. A drop of the fixed culture was put on a collodion membrane which was on the surface of an agar plate. After allowing time for the soluble components in the drop to filter through the membrane, the membrane was lifted and floated on the surface of a staining solution of 1% potassium phosphotungstate (pH 7.0). Grids were dropped onto the floating membrane, lifted, dried, and examined in a Hitachi HU-11B-1 microscope.

Counting radioactivity. ^{32}P radioactivity in solution was counted by adding 0.1-ml volumes of ether-extracted samples to 5 ml of scintillation fluid (5 g of 2,5-diphenyloxazole, 80 g of naphthalene, 360 ml of toluene, 360 ml of dioxane, and 216 ml of absolute ethyl alcohol). ^{32}P activity in precipitated material was

counted by collecting the precipitates on fiber glass filters, drying the filters, and adding the filters and 0.1 ml of water to the scintillation fluid. For the controls, the amount of radioactivity in ^{32}P -labeled phage was determined by adding 0.1 ml of phage suspension directly to the scintillation fluid. Counting was done in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

When cells were infected at multiplicities of less than 1 phage per cell in the presence of actinomycin D, approximately 90% of the infectious centers were lost irreversibly (Table 1). Actinomycin had its greatest effect when it was added to the cells before or along with the infecting PBS1 phage. The loss of infectious centers diminished when the addition of actinomycin was delayed. When actinomycin was added later than 4 min after the phage, no infectious centers were lost.

The loss of infectious centers did not result from killing of the host cells or from inactivation of free phage by actinomycin. The number of viable cells and the number of free phage remained the same after 1 hr of exposure to 27 μg of actinomycin per ml (Table 2). Indeed, cells remained completely motile in the presence of actinomycin.

TABLE 1. *Effect of actinomycin on PBS1 infection^a*

Time of actinomycin addition (min)	PFU
0	4.4×10^8
2	1.4×10^8
4	3.3×10^8
6	5.7×10^8
8	10^7
No actinomycin added	4.3×10^8

^a PBS1 phage at a PBR of 0.1 was added to 2×10^8 cells/ml in ETY medium. At the times indicated, actinomycin was added to a final concentration of 2.7 μg /ml. The cultures were incubated at 37 C with aeration for 10 min and then were sampled for the number of PFU present.

TABLE 2. *Effect of actinomycin on uninfected cells and on free phage^a*

Determination	CFU	PFU
Minus actinomycin...	3.8×10^8	1.9×10^9
Plus actinomycin...	3.2×10^8	2.3×10^9

^a Actinomycin (27 μg /ml) was added to PBS1 phage and to *B. subtilis* 168 cells in EMT medium. The cultures were assayed for PFU and for colony-forming units (CFU) just before the addition of actinomycin and after 60 min of aeration at 37 C with actinomycin.

Effect of actinomycin on phage adsorption. The amount of adsorption was determined by centrifuging host cells and adsorbed phage and then determining the number of infectious centers remaining in the supernatant medium. Actinomycin did not affect the adsorption of PBS1 to host cells. Approximately 96.5% of 2.2×10^8 infectious centers were removed from the medium with the bacteria after 10 min of adsorption. In the presence of 27 μ g of actinomycin per ml, 97% of the infectious centers were removed with the bacteria after 10 min of incubation (Table 3). The phage removed with the bacteria were resuspended and plated on a lawn of sensitive cells (Table 3). There was, however, no recovery of infectious centers when the cells infected in actinomycin were resuspended and plated. Thus, infection with PBS1 phage was aborted by actinomycin at some point after the adsorption of the virus to the host cells.

Ejection of DNA in actinomycin. Electron micrographs of PBS1 phage that had adsorbed to host cells in the presence of actinomycin were examined to determine whether the phage were able to eject their DNA. A tabulation of 342 phage seen to be in contact with the host cells showed that 17.2% were devoid of DNA (Table 4). Of 327 phage adsorbed to cells in the absence of actinomycin, 22.9% were devoid of DNA.

TABLE 3. *Effect of actinomycin on PBS1 phage adsorption^a*

Determination	PFU		
	Precipitate	CHCl ₃ -treated precipitate	Supernatant fluid
<i>B. subtilis</i> 168 . . .	2.7×10^8	2.5×10^6	7.8×10^6
<i>B. subtilis</i> 168 + PBS1 + actinomycin . . .	9.2×10^6	2.8×10^6	6.6×10^6
<i>B. subtilis</i> SB171 + PBS1		1.6×10^7	2.0×10^8
<i>B. subtilis</i> SB171 + PBS1 + actinomycin . . .		4.6×10^7	

^a Approximately 2.2×10^8 PFU of PBS1 phage were added to 4×10^8 *B. subtilis* 168 or SB171 cells. The cells were in ETY medium or in ETY medium + 27 μ g of actinomycin per ml. The cultures were incubated with aeration at 37 C for 10 min, chilled, and centrifuged at $8,000 \times g$ for 10 min. The supernatant medium was removed and assayed for PFU. The precipitated material was resuspended to its original volume in ETY medium, and the number of PFU was determined before and after treating the resuspended material with chloroform.

TABLE 4. *Effect of actinomycin on PBS1 phage ejection seen in electron micrographs^a*

Determination	Per cent ejected		Per cent tail contracted		Per cent unaltered	
	Cell walls	Flagella	Cell walls	Flagella	Cell walls	Flagella
Control	18.3	4.6	51.4	7.0	9.8	8.9
Actinomycin . . .	14.0	3.2	56.1	5.9	9.9	10.8

^a Approximately 2×10^9 PFU of PBS1 were added to 7.6×10^8 cells in ETY medium or in ETY medium + 27 μ g of actinomycin per ml. The cultures were aerated at 37 C for 10 min and then fixed with 0.5% Formalin. The fixed culture was prepared for electron microscopy. We counted 327 phage in the control cultures and 342 in the actinomycin cultures. Only those phage seen to be in contact with the cells were counted.

These data indicate that actinomycin does not inhibit the ejection of the DNA of adsorbed phage.

The usual distribution of phage on the host cells is noteworthy. Previous reports (2, 8) showed that the primary site of PBS1 adsorption is the flagella. Our tabulation shows 70 to 80% of the phage on the cell walls. The location of most of the phage on the cell walls (Fig. 1), rather than on the flagella, may be an artifact or it may reflect one of the differences between our procedures and those used in other laboratories. For instance, our phage stock was not pelleted during its preparation. As described above, CsCl was added directly to the crude lysate, and the phage were collected from the band visible in the CsCl gradient after centrifugation. When phage from a stock that had been pelleted were used to infect host cells, only 4 in 100 phage were seen adsorbed to the cell walls, and the other 96% of the phage were adsorbed to the flagella (Fig. 2).

Another difference between pelleted and non-pelleted phage was their "specific activities." Specific activity in this case is the ratio of infectious units to bacteria-immobilizing units in the phage stock. In a crude lysate this ratio was between 5 and 6. In a preparation that had been pelleted, the ratio was between 0.6 and 1.0. In a nonpelleted preparation it was about 5. Allowing for a large error due to the difficulty of judging, under a microscope, the per cent of a population of motile cells that are immobilized, it is clear that the pelleted phage suffered about a 5- to 10-fold greater loss of plaque-forming units than the nonpelleted phage.

Since the ejection of phage DNA was apparently not affected by actinomycin, we considered the possibility that actinomycin prevented the

FIG. 1. (A and B) Nonpelleted PBS1 phage adsorbed to *B. subtilis* SB19 cells in the presence of 2.7 µg of actinomycin D per ml. The PBR in this preparation was 3. (C and D) Nonpelleted PBS1 adsorbed to SB19 cells in the absence of actinomycin D. Note the affinity of the end of the phage tail core for the flagella. The distribution on the cells and the morphological alterations of the adsorbed phage are shown in Table 4.

FIG. 2. (A and B) Pelleted PBS1 phage adsorbed to *B. subtilis* SB19 cells in the absence of actinomycin D. The PBR in this culture was about 2. (C) Free PBS1 phage. (D) PBS1 phage fixed with 0.5% Formalin. The Formalin distorts the phage heads but does not cause any noticeable loss of DNA from the phage.

uptake of the DNA into the cell. The fate of phage DNA was determined by labeling the DNA with ^{32}P and then measuring the amount of P^{32} released into the medium of cells infected in the presence and in the absence of actinomycin. The P^{32} background in these experiments was estimated by adding labeled phage to SB171 cells which are not infected by PBS1 (2). The SB171 cells plus phage were incubated for 10 min, chilled, treated with cold 10% trichloroacetic acid, and centrifuged at about $8,000 \times g$ for 10 min. About 23 to 30% of the total ^{32}P radioactivity remained in the supernatant medium. This ^{32}P background was subtracted from the counts found in the medium of control and actinomycin-treated cells. We found that between 1.5 and 7.2% of the phage DNA was released during normal infection. Between 15.3 and 22.4% of the phage DNA was released into the medium when infection took place in actinomycin (Table 5). The amount of DNA released into the medium in the presence of actinomycin was insufficient to support the assumption that aborted phages ejected all of their DNA into the medium. The amount of DNA found in the medium, however, was sufficient to account for the loss of infectious centers if only a part of the genome of each aborted phage was released into the medium. This might occur if, as in the cases of T5 or T4 phage infections in KCN, the DNA was ejected from the phage but not completely fixed in or on the actinomycin-inhibited host cells.

Transduction in actinomycin. If actinomycin caused the loss of infectious centers by preventing the uptake of phage DNA into the cell, one would expect that PBS1-mediated transductions would also be lost in the presence of actinomycin, unless the transducing DNA itself specified an alternate pathway for entering the host cells. When the effect of actinomycin was tested on the PBS1 transduction of the *trp-2* locus, we found a 94% loss of transductions (Table 6).

Other inhibitors. One might assume that infecting PBS1 phage act like T5 phage to induce the production of enzymes which are responsible for the completion of DNA uptake into the cells (3). Actinomycin might then inhibit penetration by preventing the formation of messenger ribonucleic acid required for the synthesis of new enzymes (3a). If this were the way inhibition occurred, we would expect other inhibitors of protein or ribonucleic acid synthesis to abort infection centers. However, neither 8-azaguanine nor chloramphenicol caused abortion of either PBS1 infectious centers or of PBS1-mediated transductions (Table 6).

Effect of actinomycin on other *B. subtilis* phages. Phage SP10, unlike PBS1, adsorbs directly to the host cell walls. It is, like PBS1, a transducing phage, although its properties as a transducing phage differ from those of PBS1 (2a). We found that actinomycin also caused the abortion of SP10 infection (Table 7).

The virulent, nontransducing phages SP01

TABLE 5. Location of PBS1 ^{32}P -DNA ejected in actinomycin^a

Determination	PFU		Per cent PFU adsorbed	Total ^{32}P counts per min per ml	Per cent of total ^{32}P		
	Cell pellet	Supernatant fluid			Washed pellet	Supernatant fluid	Trichloroacetic acid-soluble in supernatant fluid
<i>B. subtilis</i> 168 + deoxy-ribonuclease + PBS1	3.2×10^8	2.8×10^7	94.5	1,202	31.2	42.5	35.9
	3.0×10^8	5.1×10^6	99.5	1,368		36.4	32.9
	2.0×10^8	1.4×10^7	98.0	2,038		42.1	24.8
<i>B. subtilis</i> 168 + deoxy-ribonuclease + actinomycin + PBS1	1.9×10^7	1.1×10^7	97.8	1,217	26.3	47.1	45.3
	8.7×10^6	4.6×10^6	99.5	1,262		44.3	42.6
	1.2×10^7	2.2×10^7	97.0	1,458		58.9	45.7
<i>B. subtilis</i> SB171 + deoxy-ribonuclease + PBS1		2.9×10^8	44.1	1,200	4.1	62.3	30.0
		2.9×10^8	52.0	1,450		62.7	25.7
		4.3×10^8	30.0	1,957		75.3	23.3

^a Approximately 6.1×10^8 ^{32}P -labeled phage were added to 3×10^8 cells in ETY medium with the constituents indicated. After 15 min of aeration at 37 C, the cultures were centrifuged at $8,000 \times g$ for 10 min. The supernatant medium was assayed for PFU, ^{32}P radioactivity, and ^{32}P radioactivity that remained soluble in 10% cold trichloroacetic acid. The pelleted material was resuspended to its original volume and assayed for PFU, or washed on a membrane filter and counted for ^{32}P activity. The results of three separate experiments are shown.

TABLE 6. *Effect of inhibitors on PBS1 infection and transduction*

Determination	PFU		Transductants ^a
	In pellet ^a	In culture ^a	
<i>B. subtilis</i> 168 + PBS1.....	9.9×10^7	1.2×10^8	4,640
<i>B. subtilis</i> 168 + PBS1 + actinomycin.....	5.1×10^6	1.2×10^7	270
<i>B. subtilis</i> 168 + 8-azaguanine + PBS1.....	2.1×10^8	1.2×10^8	4,530
<i>B. subtilis</i> 168 + chloramphenicol + PBS1.....	3.3×10^7	3.8×10^7	4,180

^a The PFU in pelleted cells and in an infected culture and the transductants were determined in separate experiments. In all experiments, *B. subtilis* 168 (*trp*-2⁻) cells were incubated with 2.7 μ g of actinomycin per ml, 50 μ g of 8-azaguanine per ml, or 20 μ g of chloramphenicol per ml for 5 min, diluted, and assayed for tryptophan transductants or PFU. The PBR in the PFU experiments was 1. In the transduction experiment, the PBR was 3.

TABLE 7. *Effect of actinomycin on ϕ 29, SP01, and SP10 phage infections^a*

Determination	PFU/ml
<i>B. subtilis</i> 168 + ϕ 29.....	1.3×10^7
<i>B. subtilis</i> 168 + ϕ 29 + actinomycin.....	1.5×10^7
<i>B. subtilis</i> 168 + SP01.....	5.9×10^6
<i>B. subtilis</i> 168 + SP01 + actinomycin.....	5.5×10^6
<i>B. subtilis</i> 168 + SP10.....	9.7×10^6
<i>B. subtilis</i> 168 + SP10 + actinomycin.....	8.5×10^5

^a The phages were added to 2×10^8 cells in ETY medium or in ETY medium + 2.7 μ g of actinomycin per ml. After 10 min of incubation with aeration at 37 C, the cultures were diluted and assayed for PFU. Samples of the cultures were also treated with chloroform and then assayed. All of the chloroform-treated samples had less than 10^4 surviving PFU.

and ϕ 29, which also adsorb directly to the host cell walls, were not at all affected by actinomycin (Table 7).

DISCUSSION

These experiments show clearly that PBS1 phage infection is aborted if actinomycin is present during the first 4 min of infection. The electron micrographs indicate that actinomycin does

not inhibit the ejection of DNA from the phage, whereas the ³²P labeling experiments indicate that actinomycin does inhibit the uptake of the ejected DNA into the host cells. Abortion in actinomycin is apparently not due to inhibition of synthesis of new enzymes, since chloramphenicol and 8-azaguanine do not cause abortion of PBS1 infections.

Actinomycin might act by forming complexes with the ejected phage DNA at the cell membrane so that the DNA is not taken up by the cell. This is consistent with the observation that transformation of competent *B. subtilis* cells is aborted if actinomycin is present at the time transforming DNA is added to the cells (Reiter and McClain, *in preparation*). However, this complex must be relatively easily reversed since transforming DNA, reacted with actinomycin, transforms normally immediately after the actinomycin is diluted to a low concentration. Müller and Crothers (4) have indeed shown that certain forms of DNA-actinomycin complexes are easily reversed by dilution.

Our data show that only 1.5 to 7.2% of the phage DNA was released in acid-soluble form into the medium during normal infection, but that 15 to 22% of the phage DNA was released in acid-soluble form in the actinomycin cultures. Phage DNA is not susceptible to nucleases in the medium during normal infection (W. B. Pritikin, Ph.D. Thesis, Univ. of California, Los Angeles, 1967). The phage DNA in the actinomycin cultures, therefore, appears to have been made accessible to the deoxyribonuclease added to the medium. We cannot, however, exclude the possibility that the DNA was degraded on the cell surface by cellular nucleases.

An indication that the events occurring at the cell surface during phage penetration are not simple is the immunity to actinomycin of SP01 and ϕ 29 infections. The penetration of these phages is obviously different from PBS1 and SP10 penetration. Whether this difference reflects the nontransducing or the virulent properties of these phages, or the fact that the DNA of these phages may exist as a single molecule rather than as several molecules, is not clear.

Our electron micrographs show that most of the PBS1 phages are on the cell walls, that an unusually high proportion of these (87.7%), have contracted tail sheaths, and that fully 23% are ghosted. This supports the hypothesis that PBS1 phage adsorb first to the flagella and are then transferred to the cell walls (8).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant CA-10338-02 from the National Cancer Institute.

We thank Chuen-Mo To and Robert Mackay for taking the electron micrographs.

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