Penetration of a Bacteriophage into *Bacillus subtilis*: Blockage of Infection by Deoxyribonuclease

ROBERT M. ZSIGRAY,¹ AARON L. MISS, AND OTTO E. LANDMAN

Biological Sciences Laboratories, Fort Detrick, Frederick, Maryland 21701, and Department of Biology, Georgetown University, Washington, D.C. 20007

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Plaquing of a newly isolated phage of *Bacillus subtilis*, phage 41c, is only 2% efficient in agar containing 200 μ g of deoxyribonuclease per ml. Timed deoxyribonuclease addition experiments showed that phage development is blocked in 90% of the cells if deoxyribonuclease is present during adsorption (zero-time samples), whereas 10 min after adsorption the enzyme has little effect (10-min samples). The fate of ³²P-deoxyribonucleic acid label of phage 41c in zero-time samples was compared to that in 10-min samples. In both, about 80% of the label remained with the phage-bacterium complex on initial centrifugation. However, four successive washings removed 90% of the ³²P from the zero-time samples but only 25% from the 10-min samples. In both samples, most of the washed-out label was of low molecular weight. When the time course of interruption of infection by blending was compared with interruption by deoxyribonuclease treatment, the two processes exhibited similar kinetics. It is postulated that both processes block injection at the same site, namely, the point of contact between phage tail and cell wall surface. Partitioning of ³²P label during protoplasting of zero-time and 10-min samples was similar to that observed during washing. For the protoplasting experiments, a quantitative method for plaquing protoplasts was developed. A single bacillus made of several cells can give rise to several protoplast plaque-forming units. Strain 41c was the only phage of seven tested to be inhibited by deoxyribonuclease. No other deoxyribonuclease-sensitive phages have been described.

A variety of substances are known to prevent the injection or penetration of phage nucleic acids into their hosts (3, 7, 8, 12-14, 17, 18, 21, 22). These antiviral substances include complex molecules such as enzymes (18, 21, 22) and antibody (13), and also antibiotics (3, 7, 17), and simple ions (1, 8, 12, 14, 18).

One agent useful in the investigation of mechanisms of phage injection and penetration is ribonuclease (18, 21, 22). In their study of phage f2, Zinder and Loeb found that ribonuclease effectively blocks infection of *Escherichia coli* by this small spherical virus (22). f2 phage attach to the male F pili and inject their ribonucleic acid (RNA) through the pilus into the host. Presence of 3 μ g of ribonuclease per ml in the medium blocks plaque formation by 10¹⁰ f2 particles. The enzyme inhibits phage de-

velopment when present during adsorption; however, when it is added 5 min after adsorption, no inhibition of lysis occurs. The ribonuclease-sensitive stage begins 1.5 min after phage adsorption (21); at this time the infecting RNA is "naked" within the pilus and evidently susceptible to enzymatic degradation; more than 60% of the plaque-forming units (PFU) are destroyed by the ribonuclease treatment (21).

Haas and Yoshikawa (4) have reported that deoxyribonuclease may inhibit transduction of *Bacillus subtilis* by the defective phage PBSH. No certain distinction could be made in these experiments between transduction and transformation however. In the present paper, we describe a deoxyribonuclease-sensitive step in the infection of *B. subtilis* by phage 41c and the probable site where deoxyribonuclease exerts its antiviral effect. (Portions of this

¹ Present address: Department of Microbiology, University of New Hampshire, Durham, N.H. 03824.

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MATERIALS AND METHODS

Host. The host bacterium was *B. subtilis* 168 *trp*⁻*str*^r, a streptomycin-resistant strain derived from 168 *trp*⁻ by transformation.

Phage. Phage 41c was isolated from soil by an enrichment technique (19). It was used for almost all of this work. Phages SP 3, SP 8*, SP 81, ϕ 1, ϕ 25, ϕ 29, and T ϕ 8A were obtained from R. Moyer.

Media. TYS broth contained the following ingredients per liter (adjusted to pH 6.9): tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 10 g; CaCl₂, 0.01 M; MgCl₂, 0.01 M. TP medium (5) used for preparing ³²P-labeled phage was adjusted to pH 7.0 and supplemented with 0.01 M CaCl₂. Media used for protoplast formation were SL 2 (6) and TYS broth supplemented with sucrose to a final concentration of 0.8 M.

Solid media contained the same ingredients as TYS broth with the addition of 1.5% agar (Difco) for routine platings and 0.7% agar for soft-agar overlays. Where infected protoplasts were to be scored, TYS agar was supplemented with sucrose to a final concentration of 0.5 M. DP medium plates (11) were used for plating protoplasts.

Preparation of host cell suspensions. Spores of *B. subtilis* 168 *trp*⁻*str*^r were spread on TYS agar and incubated overnight at 37 C. The growth from a plate was suspended in 2 ml of TYS broth. This suspension was used to inoculate TYS broth to an optical density at 670 nm (OD) of 0.05 (Bausch & Lomb Spectronic 20 spectrophotometer). The broth culture was shaken at 37 C until it reached an OD of 0.2. The cells were then centrifuged and resuspended in one-fourth volume of fresh TYS broth to a final concentration of 1×10^8 to 2×10^8 colony-forming units/ml (CFU/ml). These suspensions are designated log-phase or concentrated cultures.

Phage titrations. Phage dilutions were made in plain or sucrose-supplemented TYS broth; 0.1 ml of the final dilution was pipetted onto plain or supplemented TYS agar plates. Five milliliters of melted soft agar at 45 C, which had been seeded with the vegetative or the spore form of the host (approximately 10^8 CFU/ml), was then overlaid onto the agar plates and thoroughly mixed with the phage. All plates were incubated either at 37 or 30 C for 12 to 18 hr.

Timed deoxyribonuclease treatments. Deoxyribonuclease I (EC 3.1.4.5, Worthington Biochemicals, Freehold, N.J.) at a final concentration of 200 μ g/ml, was added either before the phage or at various times during infection. In the former case, the nuclease was added to one of two tubes containing 5 ml of a concentrated bacterial culture. Phage 41c was added to both tubes at a multiplicity of infection (MOI) of less than one, and the samples were placed on a Burrell wrist-action shaker for 10 min at 30 C. An equivalent amount of the enzyme was then added to the tube

without deoxyribonuclease, and incubation was continued for an additional 10 min. Next, the infected complexes were diluted and assayed for PFU.

To start an experiment involving timed addition of deoxyribonuclease to infected cultures, phage 41c at an MOI of less than one was added to 5 ml of a log-phase culture at 5 C. After an adsorption period of 30 min at 5 C, samples of the mixture were withdrawn and diluted into plain TYS broth and TYS broth containing 200 μ g of deoxyribonuclease per ml. The remaining adsorption mixture was warmed with shaking to 30 C. At 3, 6, and 10 min, additional samples were withdrawn and diluted into plain TYS broth and TYS broth containing deoxyribonuclease. Incubation was continued at 30 C until each deoxyribonuclease-treated sample had been exposed to the nuclease for a period of 10 min. The infected cultures were then diluted and assayed for PFU. When appropriate, subsamples were treated with chloroform to determine the number of free phage.

Survival of protoplasts in various hypertonic media. Log-phase cultures of the host were resuspended in SL 2 medium or in sucrose-supplemented TYS broth. A viable count was made, and lysozyme at a final concentration of 200 μ g/ml was added to each sample. The suspensions were then incubated at 32 C in shallow layers (5 ml in 125-ml Erlenmeyer flasks) without agitation until protoplast formation was complete. They were then diluted in the homologous medium and plated on DP medium. All plates were incubated at 30 C for 36 hr.

Plaques of infected protoplasts. A log-phase culture of the host was prepared, and phage 41c was added to an MOI of approximately 0.1. The mixture was placed on the wrist-action shaker and agitated for 10 min at 30 C to permit phage penetration. The infected culture was then transferred to a 125-ml Erlenmeyer flask and supplemented with sucrose to a final concentration of 0.8 M. Chloramphenicol at a final concentration of 20 μ g/ml was added to block further phage maturation. A sample was withdrawn, diluted in TYS-sucrose broth, and plated for PFU. Lysozyme was then added to the experimental flask to a final concentration of 200 μ g/ml, and the culture was incubated statically in a water bath at 32 C until protoplast formation was complete. Samples of the infected protoplasts were diluted in TYS-sucrose broth, and, as a test for osmotic fragility, in physiological saline, and 0.1-ml amounts of the appropriate dilutions were delivered to TYS-sucrose plates. Five milliliters of melted TYS-sucrose agar (at 45 C), which had been seeded with approximately 10^8 vegetative cells, was overlaid onto the hard agar and thoroughly mixed with the infected protoplast sample. All plates were incubated either at 30 C or at room temperature for 18 to 24 hr.

³²P-labeling of phage 41c. The host was grown in TYS broth to an OD of 0.2 and centrifuged at $10,000 \times g$ for 5 min. The pellet was resuspended to the original volume of 5 ml in TP medium, and a total of 0.1 mCi of ³²P (International Chemical and Nuclear Corp.) was added. The culture was infected with phage at an MOI of approximately 2, and the mixture was shaken at 26 C for 150 min. The lysate was cen-

trifuged at $10,000 \times g$ for 5 min, the supernatant fluid was filtered (Millipore filters, type HA, mean pore size $0.45 \mu\text{m}$), and the filtrate was collected. Deoxyribonuclease and ribonuclease were added to final concentrations of $50 \mu\text{g/ml}$, and the mixture was incubated at 37°C for 30 min. The lysate was then centrifuged at $89,000 \times g$ in a Beckman model L ultracentrifuge for 120 min. The supernatant fluid was decanted, and the pellet was washed three times by gentle rinsing with 10 ml of TYS broth. The pellet was then resuspended in TYS broth to the original volume.

Assay for radioactivity. The scintillation fluid used for counting the ^{32}P -labeled samples contained the following constituents: Triton X-100 (Packard), 166 ml; 2,5-diphenyloxazole (Packard), 1.0 g; 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (Packard), 100 mg; deionized distilled water, 1,000 ml. This mixture was stirred for 60 min and filtered through Whatman no. 1 filter paper. The labeled samples (1.0 ml) were added to vials containing 20 ml of scintillation fluid and counted either in a Nuclear-Chicago liquid scintillation spectrometer (systems 724-725) or a Packard Tri-Carb liquid scintillation spectrometer model 3310.

Release of adsorbed label during protoplast formation. A 10-ml volume of a concentrated culture was divided in two. Deoxyribonuclease at a final concentration of $200 \mu\text{g/ml}$ was added to one of the two samples, and both were infected with ^{32}P -labeled phage 41c at an MOI of approximately 1.0. The mixtures were placed on the wrist-action shaker for 10 min at 30°C , and an equivalent amount of deoxyribonuclease was then added to the sample lacking the enzyme. After an additional incubation period of 10 min at 30°C , chloramphenicol at a final concentration of $50 \mu\text{g/ml}$ was added to both phage-bacterium mixtures to stop further phage maturation, and the samples were centrifuged at $10,000 \times g$ for 5 min. The supernatant fluids were collected, and the pellets were resuspended in 5 ml of TYS broth containing $50 \mu\text{g}$ of chloramphenicol per ml. A concentrated sucrose solution containing $50 \mu\text{g}$ of chloramphenicol per ml was added to obtain a final concentration of 0.8 M. A sample of each culture was diluted in TYS-sucrose broth and assayed for PFU on TYS-sucrose agar. In addition, 0.2 ml of each culture was assayed for radioactivity.

The undiluted infected complexes in sucrose-chloramphenicol medium were then transferred to 125-ml Erlenmeyer flasks, and lysozyme was added to a final concentration of $200 \mu\text{g/ml}$. Incubation in a water bath at 32°C followed until protoplast formation was complete. To check on the survival of infective centers, samples of the infected protoplasts were assayed for PFU on TYS-sucrose agar. Other samples were chloroform-treated to determine the number of free phage.

The infected protoplasts were then centrifuged at $10,000 \times g$ for 10 min, and the supernatant fluids were collected. The protoplast pellets were resuspended to the original volume with TYS-sucrose broth, and 1 ml of each sample (both protoplasts and supernatant fluids) was added to scintillation vials

and assayed for radioactivity.

RESULTS

Characterization of phage 41c. The morphology of phage 41c was examined by negative staining (2). The phage head is hexagonal (icosahedral?) and approximately 50 nm in diameter. The tail is 140 nm long and 10 nm in diameter. Tail fibers, baseplates, and contractile sheaths are not discernible in our electron micrographs.

Estimates of the guanine plus cytosine (G + C) content of the deoxyribonucleic acid (DNA) of phage 41c were obtained by thermal denaturation (9) and by cesium chloride centrifugation (16). Both techniques agreed in showing that the mole percent G + C of phage 41c is the same as that of *B. subtilis*. It thus appears unlikely that the phage DNA contains a substantial proportion of any unusual bases.

Preliminary observations on nuclease inhibition. In our initial experiments, infection with 41c (MOI approximately 0.2) was permitted to proceed in the presence of a mixture of $200 \mu\text{g}$ each of deoxyribonuclease and ribonuclease per ml. A comparison of the number of plaques obtained in the presence and absence of the enzyme mixture revealed a loss of more than 90% of the PFU in the nuclease-treated samples. To determine which of the enzymes was responsible for the inactivation of the phage, they were tested separately. This time, the deoxyribonuclease-treated sample showed a substantial loss of PFU, whereas there was little if any decrease in plaque number due to ribonuclease (Table 1). The inhibitory effect of various concentrations of deoxyribonuclease in the plating medium on plaquing by 41c is shown in Table 2. Since nearly complete inhibition was reached only at $200 \mu\text{g}$ of deoxyribonuclease, this concentration of enzyme was adopted as standard.

How does deoxyribonuclease block phage development? Several mechanisms could account for the inhibition of plaquing by deoxyribonuclease. (i) Free virus—or even the host—might be sensitive to $200 \mu\text{g}$ of the enzyme per ml. (ii) Deoxyribonuclease might block adsorption of the phage. (iii) The phage genome

TABLE 1. Plaquing of phage 41c after infection in the presence of ribonuclease or deoxyribonuclease

Host-phage mixture supplemented with:	Percent recovery
Deoxyribonuclease, $200 \mu\text{g/ml}$	28
Ribonuclease, $200 \mu\text{g/ml}$	96
No nuclease	100 ^a

^a 100% recovery corresponds to 3.2×10^7 PFU.

TABLE 2. *Effect of various concentrations of deoxyribonuclease on the plating efficiency of phage 41c*

DNase concn in the agar ($\mu\text{g/ml}$)	Percent recovery
0	100.0 ^a
10	39.4
25	25.3
50	12.9
100	8.6
200	2.2

^a 100% recovery corresponds to 1.7×10^{11} PFU.

might become accessible to nuclease attack in the course of infection.

The first of the foregoing possibilities was easily ruled out. When either host or phage was incubated with 200 μg of deoxyribonuclease per ml for 10 min at 30 C, there was no loss in either CFU or PFU; clearly, deoxyribonuclease does not inactivate *B. subtilis* or free phage. The second possibility—inhibition of adsorption by deoxyribonuclease—could be similarly eliminated. By using standard phage techniques (1) it was shown that more than 95% of 41c phage were adsorbed to the host, regardless of whether deoxyribonuclease was present during adsorption. Moreover, less than 1% of the phage-bacterium complexes formed in the presence of deoxyribonuclease survived chloroform treatment. Evidently, the enzyme does not prevent the initiation of infection after adsorption. To investigate the third possibility—that the phage genome might become accessible to nuclease in the course of infection—the effect of deoxyribonuclease upon successive stages of infection was studied. Phage-bacterium complexes (preadsorbed at 5 C) were transferred to deoxyribonuclease-TYS broth at various times following warming to 30 C; then plaque formation in the nuclease-treated samples was compared with plaque formation in untreated controls (Table 3). It is evident that a sharp loss of PFU occurred when the enzyme treatment was given at zero time (without prior warming) or 3 min after warming. Even 6 min after transfer to 30 C, 43% of the PFU could still be inactivated by deoxyribonuclease; after 10 min at 30 C, however, most of the complexes were no longer sensitive.

The effect of deoxyribonuclease treatment is particularly clear-cut if the enzyme is added *before* the phage. Under these circumstances, well over 90% of the phage were inactivated (Table 4); chloroform survival data show that over 99% of the phage had initiated injection “at” zero time (Table 4). In view of these clear

results, the practice of adding deoxyribonuclease prior to the phage for the zero-time sample was adopted in subsequent tracer studies of the deoxyribonuclease effect (*see below*).

A protoplast system for the study of phage DNA penetration: viability of protoplasts as CFU and PFU. The preceding results indicated that inactivation of the phage by deoxyribonuclease occurred during or after adsorption—presumably between the time 41c began to eject its DNA and the time this DNA entered into deoxyribonuclease-inaccessible sites in the recipient bacterium. To pinpoint the site of attack of deoxyribonuclease on the penetrating DNA, the effect of cell wall removal on the infectious process was investigated. Analogous studies on the fate of transforming DNA in *B. subtilis* during wall removal have been useful in the analysis of the successive steps in transformation (10, 20).

A prerequisite for such experimentation was the development of a hypertonic medium that would permit the survival of virtually all protoplasts made from the phage-sensitive log-phase cultures. With this aim in mind, protoplasting experiments were performed with log-phase cultures suspended in SL 2 medium and in TYS broths supplemented with various concentrations of sucrose, and the efficiency of plating of the protoplasts was tested (as L-colony-forming units [L-CFU] on DP medium). The results of two such experiments are shown in Table 5. It will be noted that maximum viabilities were obtained in TYS broth supplemented with 0.75 M sucrose. Because nearly complete recoveries of *B. subtilis* protoplasts from stationary-phase cultures can be obtained in SL 2 medium (6, 11), these data suggest that protoplasts from log-phase cells are osmotically more sensitive. Table 5 also shows that more than one protoplast originated from a single bacillary CFU (B-CFU). When the log-phase bacteria were examined in a phase microscope, they were found to be in short chains; a survey of 40 bacteria gave an average of three cells per chain. This observation explains the fact that the recovery of L-CFU could reach 282% in TYS-sucrose broth (Table 5).

Having established the suitability of TYS-sucrose broth for stabilizing host cells in the protoplasted state, we then devised a procedure for scoring infected protoplasts. The host was infected with phage 41c (MOI of 0.1) and incubated for 10 min at 30 C, the walls were removed, and the protoplasts were plated as PFU on TYS-sucrose layer plates. As shown in Table 6, the number of PFU was the same before and after protoplast formation. In addition, more

TABLE 3. *Inhibition of infection by deoxyribonuclease: dependence upon time of nuclease addition^a*

Time of DNase addition	PFU recovered from			(d) Complexes that initiated infection (a - b)	(e) DNase-resistant complexes (c - b)	(f) DNase-sensitive complexes (d - e)	Inactivation by DNase of injected 41c (%) (f/d × 100)
	(a) Control (no enzyme)	(b) CHCl ₃ treatment	(c) DNase treatment				
0	3.7×10^7	1.7×10^7	2.1×10^7	2.0×10^7	4.0×10^6	1.6×10^7	80
3	3.6×10^7	4.2×10^6	1.0×10^7	3.2×10^7	6.0×10^6	2.6×10^7	81
6	4.0×10^7	3.3×10^5	2.3×10^7	4.0×10^7	2.3×10^7	1.7×10^7	43
10	4.0×10^7	1.0×10^5	3.7×10^7	4.0×10^7	3.7×10^7	3.0×10^6	8

^a Phage-bacterium complexes (at 5 C) were warmed to 30 C and diluted into the appropriate broths at the indicated times. Each sample was incubated at 30 C for an additional 10 min and assayed. Under the standard conditions of incubation at 30 C, the complexes begin to burst 60 min after infection.

TABLE 4. *Inhibition of infection by deoxyribonuclease: enhanced inhibition when phage is added in presence of deoxyribonuclease*

Expt	Time of DNase addition ^a	PFU recovered ^b	CHCl ₃ -resistant PFU	Inactivation by DNase (%)
1	0	1.2×10^7	1.7×10^5	94
	10	2.0×10^8	4.7×10^5	
2	0	3.2×10^6	$<1.0 \times 10^5$	96
	10	8.3×10^7	1.3×10^5	
3	0	7.0×10^5	$<1.0 \times 10^5$	98
	10	3.6×10^7	$<1.0 \times 10^5$	

^a 0 = deoxyribonuclease added to host before phage; 10 = deoxyribonuclease added 10 min after infection.

^b Deoxyribonuclease inactivated by 5 min of heating at 100 C retains less than 20% of the inhibitory activity; electrophoretically pure deoxyribonuclease is highly inhibitory.

TABLE 5. *Survival of protoplasts from log-phase bacilli in various hypertonic sucrose media*

Protoplasting medium	Viable counts		Percent recovery ^a	
	Expt 1 ^b	Expt 2 ^c	Expt 1	Expt 2
SL 2 (0.5 M sucrose)	1.3×10^8	1.6×10^7	118	41
TYS + 0.5 M sucrose	1.8×10^8	8.1×10^7	164	208
TYS + 0.75 M sucrose	2.5×10^8	1.1×10^8	227	282
TYS + 1.0 M sucrose	9.9×10^7	6.1×10^7	90	156

^a Percent recovery = (L-CFU after protoplast formation / B-CFU before protoplast formation) × 100.

^b The viable count before protoplast formation was 1.1×10^8 .

^c The viable count before protoplast formation was 3.9×10^7 .

than 95% of the PFU were osmotically sensitive, indicating that the viral genomes were indeed associated with protoplasts.

When infection with 41c was carried out at an MOI greater than 1 and a portion of the sample was converted to protoplast, the total number of infected protoplasts observed always exceeded the number attained in the untreated bacillary samples. Thus, as expected, more

TABLE 6. *Plaquing of phage-infected protoplasts*

Type of sample	PFU recovered	Percent recovery
Infected bacilli ^a	1.4×10^7	100
Infected protoplasts	1.5×10^7	100
Osmotically shocked protoplasts	6.0×10^5	4

^a MOI was 0.1.

than one infected protoplast can emerge from a single B-CFU.

Release of adsorbed phage DNA label from infected bacilli and protoplasts treated with deoxyribonuclease. With the availability of a technique for the physical separation of protoplasts from their (enzyme-degraded) walls and wall-attached infecting phage, we could then study the partitioning of phage DNA label between protoplasts and cell envelope in relation to deoxyribonuclease treatment. Is the loss of PFU due to deoxyribonuclease treatment correlated with an interruption of the flow of DNA between cell envelope and the protoplast interior? If so, where and how is the injection interrupted?

An experiment dealing with these questions is recorded in Tables 7 and 8. Deoxyribonucle-

ase was added to cultures of the host either before starting infection with ^{32}P -labeled phage 41c or 10 min postinfection. After an additional 10 min of incubation at 30 C, maturation in both samples was stopped by chloramphenicol. The samples were centrifuged, and portions of the resuspended pellets were assayed for PFU and adsorbed ^{32}P label. The total amount of label adsorbed was found to be essentially the same in both cultures (Table 8); however, 98% of the phage were inactivated by deoxyribonuclease in the zero-time sample (Table 7). These results indicated again that deoxyribonuclease did not interfere with the adsorption and injection processes of the virus. Since 80% or more of the added radioactivity could be centrifuged into the pellet (Table 8), extensive solubilization of the label had apparently not occurred at this time.

TABLE 7. *Effect of deoxyribonuclease treatments studied with labeled phage and protoplasting techniques: plaquing and injection before and after wall removal^a*

Nature of sample	Time of DNase addition	PFU recovered	Percent recovery	Percent injection
Infected bacilli	0	5.9×10^6	5	
	10	1.1×10^8	100	
Infected protoplasts	0	5.2×10^6	4	
	10	1.3×10^8	100	
CHCl_3 -treated protoplasts	0	4.1×10^6	3	97
	10	6.5×10^8	<1	>99

^a Deoxyribonuclease was added before infection with ^{32}P -labeled phage and 10 min postinfection. After an additional 10 min of incubation, maturation was stopped by chloramphenicol. The suspensions were centrifuged, reconstituted in 0.8 M sucrose-TYS-chloramphenicol broth, and sampled for PFU and radioactivity. The mixtures were then treated with lysozyme for 45 min, the resultant protoplasts were assayed for PFU with and without CHCl_3 treatment, and the suspensions were further fractionated for radioactivity measurements (see Table 8).

When each of the infected cultures was converted to protoplasts and reassayed for PFU, good recoveries (as compared to the intact bacillary systems) were obtained (Table 7). However, the amount of label retained by the protoplasts varied depending on the time deoxyribonuclease had been added (Table 8). When the enzyme was present before infection, only 14% of the total adsorbed label remained associated with the protoplasts; the remaining 86% was released into the medium. When the nuclease was added 10 min after infection, approximately 80% of the adsorbed label had passed into the protoplasts.

To study further the labeled material released from the zero-time sample by conversion to protoplasts, cold trichloroacetic acid was added to a sample of the protoplasting supernatant fluid to a final concentration of 5%. As shown in Table 8, only about one-fifth of the released radioactivity was found to be in cold acid-precipitable macromolecular form. Apparently, the infecting DNA of 41c in the zero-time sample is readily accessible to hydrolysis by deoxyribonuclease.

The extensive degradation of phage DNA might have occurred during the prolonged incubation for protoplast formation. To check on this possibility, we attempted to remove the enzyme before the protoplasting formation step by repeated washing (centrifugation) with TYS broth containing 25 μg of chloramphenicol per ml. The results showed that, even without protoplast formation, about 90% of the adsorbed radioactivity could gradually be washed from the zero-time samples (Table 9). By contrast, only about 25% of the adsorbed activity was washed from the 10-min samples. In both cases, less than half of the material removed by the washing was cold acid-precipitable (Table 9). It is clear, therefore, that, at least in the zero-time sample, the bulk of the phage DNA is readily

TABLE 8. *Effect of deoxyribonuclease treatments studied with labeled phage and protoplasting techniques: adsorption release and characterization of ^{32}P -labeled material^a*

Time of DNase addition	Corrected counts per min per ml associated with ^b				
	Bacilli	Protoplast pellet	Protoplast supernatant	Acid-insoluble fraction	Acid-soluble fraction
0	1,265 (1,605) ^c	180 (263)	1,038 (1,332)	270 (359)	1,135 (1,195)
10	1,210 (1,490)	951 (1,124)	242 (281)		

^a After the formation of protoplasts (see Table 7), the samples were centrifuged and the supernatant fluids were collected. The pellets were resuspended, and 1 ml of each sample (both supernatant and pellet) were assayed for ^{32}P . In addition, the protoplast supernatant fluid of the zero-time sample was precipitated with trichloroacetic acid at 0 C.

^b Based on input values of 1,518 and (1,900) counts per min per ml.

^c Figures in parentheses are data from a repeat experiment.

TABLE 9. Release of ^{32}P label during successive washings of deoxyribonuclease-treated infected complexes; characterization of released ^{32}P -labeled material^a

Time of DNase addition (min)	PFU recovered	Total counts/min not adsorbed to host ^b	Total counts/min released during wash no. ^b				Total counts/min in washed bacilli ^b
			1	2	3	4	
0	5.0×10^6	3,675	3,675	1,570	655	320	680
10	8.0×10^7	2,705	895	495	270	165	5,610
		Corrected counts/min associated with (wash no. 1)		Corrected counts/min associated with (wash no. 2)			
		Acid-insoluble fraction	Acid-soluble fraction		Acid-insoluble fraction		Acid-soluble fraction
0		1,058	2,463		750		750
10		353	513		285		250

^a After deoxyribonuclease treatment, the infected cultures were washed repeatedly by centrifugation. The total counts per minute associated with (i) unadsorbed label, (ii) each wash, and (iii) bacilli was determined. The first two washes of each of the enzyme-treated samples were analyzed by cold trichloroacetic acid precipitation.

^b A total of 11,000 counts/min was added to each system.

accessible to deoxyribonuclease attack, that the degradation of the DNA is not halted by washing, and that wall removal is not required to facilitate access of the enzyme to the DNA.

Comparison of the interruption of infection by deoxyribonuclease with interruption by blending. The preceding results show that sometime between the start of its ejaculation from the phage particle and the termination of its passage through the protoplast membrane, the DNA of phage 41c passes a site accessible to deoxyribonuclease. This site could be the area of contact between phage tail and cell wall on the wall surface or, alternatively, locations within the wall or in the periplasmic space. An approximate indication of where this nuclease-accessible site might be could be gained by comparing the kinetics of interruption of infection by deoxyribonuclease with interruption kinetics by an agent which acts at a known site. Such an agent is the blender: blending prevents infection until all essential parts of the phage genome have moved past the area of contact between phage tail and cell wall surface. The experiment exhibited in Table 10 shows that at 3 min postinfection 83% of the complexes were inactivated by blending and that at 15 min postinfection 21% of the complexes could still be destroyed by blending. Parallel data on sensitivity to deoxyribonuclease (Table 3) show 82% inactivation at 3 min postinfection and 8% inactivation at 10 min postinfection. Susceptibility to blending and deoxyribonuclease thus exhibit very similar kinetics; if anything, susceptibility to deoxyribonuclease disappears sooner than susceptibility to blending. In sum,

the kinetic comparison suggests that deoxyribonuclease acts early in infection and perhaps at the same site as blending, namely, the area of contact between phage tail and cell wall surface.

Rescue of infected complexes by deoxyribonuclease. Further insight concerning the site and mode of action of deoxyribonuclease can be gained by studying the rescue of bacilli from phage-induced death by deoxyribonuclease. On the assumption that injection of a small percentage of the genes from the virulent 41c phage would kill the host, the extensive rescue effected by preinfection addition of deoxyribonuclease (Table 11) indicates that relatively few phage genes penetrate into the host cell past the nuclease block. This conclusion is of course consonant with the tracer data (Tables 8 and 9) and accords well with the inference drawn from the blending data that the site of deoxyribonuclease action is at the cell surface.

DISCUSSION

In this study we have shown that deoxyribonuclease is a specific antiviral agent for a phage of *B. subtilis*. The phage, 41c, appears to be special in regard to this characteristic: the lytic cycles of all other *B. subtilis* phages tested, SP 3, SP 8*, SP 81, $\phi 1$, $\phi 25$ and T ϕ 8A, were not affected by deoxyribonuclease. Indeed, to our knowledge, sensitivity of phage infection to deoxyribonuclease has not previously been reported; in distinguishing transduction from transformation by the deoxyribonuclease sensitivity criterion, it has generally been assumed that the transport of DNA from the phage into

the bacterial cell is not accessible to deoxyribonuclease attack.

What is the mechanism whereby deoxyribonuclease blocks plaquing by phage 41c? The observation that deoxyribonuclease inhibition is most effective—and only effective—when the enzyme is present during adsorption or in the first minutes after adsorption clearly shows that deoxyribonuclease acts at an early stage in infection. This inference is strongly supported by the finding that 80 to 100% of the host bacteria in infected complexes can survive if infection is carried out in the presence of deoxyribonuclease (Table 11).

Since phage 41c plaques normally on a non-flagellated mutant of *B. subtilis*, we infer that the phage adsorb to the cell surface. Our results do not give us clear further guidance concerning the precise site in the cell envelope where deoxyribonuclease is acting. However, several arguments can be made in favor of the hypothesis that the site of deoxyribonuclease attack is in the area of contact between phage tail and

cell wall surface rather than in the periplasm.

(i) In order to attack the DNA in a periplasmic or intra-cell-wall site, deoxyribonuclease would have to penetrate the wall of *B. subtilis*. Experiments with transforming systems show that this does not happen ordinarily, although exceptions occur when the wall has been partly depolymerized (20). Since none of the other *B. subtilis*-phage complexes were susceptible to the enzyme, one would have to postulate that phage 41c depolymerizes the wall in a special way allowing access of deoxyribonuclease to the periplasm. In our view, a more economical hypothesis is that the tail-cell wall junction is less perfect in 41c than in other phages, allowing a small amount of diffusion of deoxyribonuclease into the junction. The requirement for very high deoxyribonuclease levels (Table 2) is consistent with this postulate.

(ii) The finding that 30 to 53% of the DNA released by washing from deoxyribonuclease-treated complexes is in cold trichloroacetic acid-precipitable form (Table 9) is readily explained by the assumption that this fraction consists of DNA still enclosed in phage heads. Several less plausible assumptions are necessary to explain the existence, distribution, and elution by washing of this fraction in terms of a periplasmic hypothesis.

(iii) When the infected complexes were subjected to high-speed blending 3 min after the start of injection, 83% of the PFU were inactivated (Table 10). This result shows that, at 3 min, genes vital to phage development were still in the protein coat in 83% of the complexes; in 17% of the cases, the entire genetic complement of the phage had entered the cell. Accordingly, breakage of the DNA at the cell surface at that moment by deoxyribonuclease or any other agent should result in 83% inactivation of the PFU. The inactivation due to deoxyribonuclease actually recorded (in a separate

TABLE 10. Effect of high-speed blending on 41c-infected complexes^a

Time of sampling (min)	Type of treatment		PFU recovered
	Blending	CHCl ₃	
3	+	—	4.2×10^6
	+	+	9.3×10^5
	—	—	1.9×10^7
15	+	—	1.5×10^7
	+	+	2.3×10^5
	—	—	1.9×10^7

^a Phage-bacterium complexes at 5 C were warmed to 30 C. Samples were withdrawn at the designated times and blended in a Sorvall Omnimixer for 5 min at top speed. A portion of each sample was chloroform-treated to determine free phage. Unblended samples served as controls.

TABLE 11. Rescue of bacilli from killing-by-infection by timely deoxyribonuclease treatments^a

Expt	Viable count/ml of uninfected bacilli	Time of DNase addition	PFU recovered	Percent PFU inactivated	CFU recovered	Percent CFU rescued
1	2.0×10^8	0	3.3×10^6	97	1.8×10^8	90
		10	1.1×10^8		3.1×10^7	16
2	2.9×10^8	0	3.0×10^6	97	2.4×10^8	83
		10	1.1×10^8		7.9×10^7	27
3	1.7×10^8	0	1.0×10^6	99	1.8×10^8	100
		10	1.2×10^8		1.4×10^7	8

^a Deoxyribonuclease was added to one of two log-phase cultures of the host. Both host cultures were infected with 41c (MOI = 1), and after 10 min of incubation at 30 C, deoxyribonuclease was added to the tube lacking the enzyme. After an additional 10 min of incubation, each infected sample was diluted and assayed for PFU and CFU.

experiment, Table 3) was 82%. Good agreement is predicted if deoxyribonuclease acts on the cell surface; it represents a notable coincidence if deoxyribonuclease acts in the periplasm.

One of the most important contributions of this paper is quite unrelated to the above considerations of the site of action of deoxyribonuclease, namely, the development of quantitative techniques for the plaquing of protoplasts (infected before wall removal). Earlier studies with spheroplasts are not comparable because spheroplasts retain phage attachment sites and are routinely prepared before infection. Moreover, quantitative survival of the spheroplasts is not usually attained. Plaquing of infected protoplasts was reported earlier, but good survival of PFU was not obtained (15).

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