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Primary Adsorption Site of Phage PBS1: the Flagellum of *Bacillus subtilis*

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The adsorption of *Bacillus subtilis* phage PBS1 was studied, and it was demonstrated that the primary adsorption site for this phage is the flagellum of *B. subtilis*. The capacity of flagella to function for motility may be lost without the loss of their capacity to adsorb the phage and permit infection. Deoxyribonucleic acid injection by the phage is inhibited by cyanide, suggesting the requirement for cellular energy in the infection process.

Joys (9) and Frankel and Joys (7) recently suggested that the receptor site for phage PBS1 (16) is the flagellum of *Bacillus subtilis*. They correlated susceptibility to phage adsorption and infection with motility of the organism, and demonstrated that four flagella-less (*fla*$^-$) mutants and one flagellated but nonmotile mutant (*mot*$^-$) of *B. subtilis* do not adsorb PBS1. They concluded that functional flagella were required for phage adsorption. The extensive investigations of Meynell (11) and of Schade, Adler, and Ris (14) have shown that the *chi* phage of *Salmonella* also exhibits flagellotropic properties.

Interest in this laboratory for the past several years has focused on the bacterial flagellum. For this reason, and because of the possible biological significance that phage adsorption on the bacterial flagellum might have, a more extensive investigation on the general properties of phage PBS1 was undertaken.

MATERIALS AND METHODS

Organisms and growth medium. B. subtilis strains SB19, SB171, 168, and SC3, 4, 6, and 23 were used in these studies. SB19 is a streptomycin-resistant prototrophic derivative of 168. SB171 is a *fla*$^-$ derivative of 168 (*trp*$^-$) obtained from E. Lederberg. Strains SC3, 4, 6, and 23 are paralyzed mutants of 168 whose flagella lack the long period helix (Martinez et al., *submitted for publication*). The other *fla*$^-$ mutants used were from our collection. The medium used for propagation and dilution of cells was TY broth (12). Bottom layers and soft agars for plating contained 1.5% and 0.6% agar in TY broth, respectively. The mineral salts solution from the minimal medium described by Spizizen (15) was used for dilution of the phages. Cultures of SB19 were grown in TY broth at 37 C to a cell density of 10^8 to 5×10^8 per ml and diluted as required. Under these conditions, more than 90% of the cell population showed translational motility. Bacteria were grown at 37 C with vigorous aeration. A modification of the assay for free phage developed by W. B. Pritkin (Ph.D. Thesis, Univ. of California, Los Angeles, 1967) was found to be the most reproducible of all those tried. One milliliter of diluted phage suspension (or infected bacteria) was mixed with 0.05 ml of indicator cells (actively motile *B. subtilis* SB 19, at a density of 5×10^8 per ml); 10 min was allowed for adsorption. Then, 0.1 ml of the mixture was added to 2.5 ml of soft agar held at 50 C; 0.15 ml of the SB19 suspension was added and this mixture was poured over the 1.5% TY agar plate as a uniform layer. Fresh bottom-layer plates were used with an adsorbent pad on the lid. The plates were incubated right side up in a humidified incubator at 26 C for 12 to 14 hr. Because of inherent difficulties in the enumeration of PBS1 particles, a free phage control was included in all experiments; this control is labeled "phage control" in the tables.

Preparation of protoplasts. Exponentially growing cultures of SB19 were harvested and washed by centrifugation with 10^{-3} M tris(hydroxymethyl)-aminomethane (Tris) buffer (*p*H 7.6). The cells were resuspended in a solution containing lysozyme (100 μg/ml), sucrose (200 mg/ml), and Tris (100 μg/ml, *p*H 8.0). The suspension was incubated at 37 C for approximately 30 min, during which time the conversion of vegetative cells to protoplasts was followed by phase-contrast microscopy. The protoplasts were sedimented by centrifugation at $5,900 \times g$ for 10 min and resuspended gently in TY broth containing 20% sucrose.

Deflagellation and flagella regeneration. Actively motile cultures of SB19 or 168 were deflagellated in the cold in a Sorvall Omnimixer for 1 min with a rheostat setting of 100 v. The cells were sedimented by centrifugation, washed once with TY broth, and resuspended in TY broth. The cell suspension was incubated at 37 C on a reciprocal shaker to allow flagella regeneration.

Phage adsorption was measured either by the chloroform method or by centrifuging the bacteria-

phage complexes at 3,600 × *g* for 15 min and assaying the supernatant solution for free phage (2).

Anti-PBS1 antiserum with a *K* value of 2,450 was a gift of W. Pritikin. Anti-flagellar antiserum was prepared by the method of Ada et al. (1) and was purified by the method of Grant and Simon (8).

Electron microscopy was performed with a Hitachi HU 11A microscope. Specimens were stained with 2% uranyl acetate.

RESULTS

Growth characteristics of PBS1. One-step growth curves of PBS1 on SB19 grown on TY broth at 37 C showed a latent period of 35 to 40 min and an average burst size of 23. These data agree well with those reported by Takahashi (16).

Figure 1 shows the kinetics of adsorption of PBS1 by SB19 at various phage to bacteria ratios (P/B). In these experiments, air was vigorously bubbled through the culture, a condition required for optimal efficiency of adsorption and infection. The adsorption rate constant calculated from the experiments with P/B of less than 1 was 1.44×10^{-8} ml/min. This rate is about 10-fold faster than that reported for the *chi* phage (11, 13) and for most *B. subtilis* phages except SP13 (4). At P/B greater than 1, the adsorption kinetics show a biphasic response. The slope of the curves after the initial 2 to 3 min of adsorption, however, depended on the P/B used.

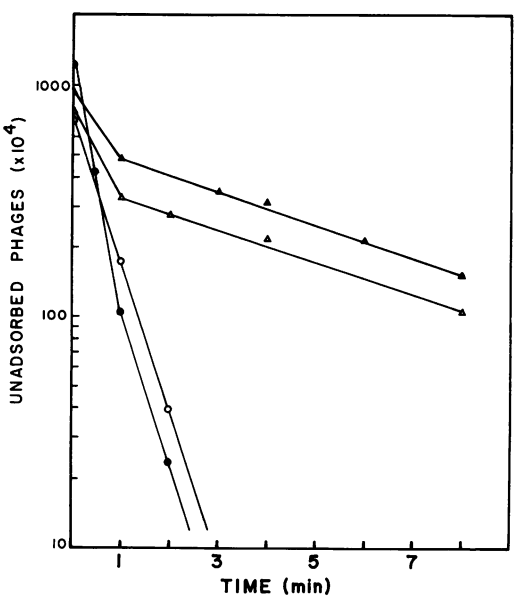


FIG. 1. *Kinetics of adsorption of PBS1 by Bacillus subtilis SB19. To actively motile SB19 cells in TY broth at 37 C phage was added at various P/B ratios and the mixture was bubbled vigorously with air. At time intervals, samples were removed and assayed for free phage by the chloroform method.* (▲) *P/B = 2.5;* (△) *P/B = 10;* (○) *P/B = 0.02;* (●) *P/B = 0.05.*

From consideration of the dimensions of the head of this phage (6), one would expect the deoxyribonucleic acid (DNA) content to be greater than that of *Escherichia coli* phage T5, and indeed a value of 2×10^8 daltons has been recently reported (5). Lanni (10) has shown that 20 to 25 min is required for formation of phage T5-bacteria complexes which are insensitive to shear. The kinetics of infection of SB19 by PBS1 were measured by deflagellating cells after phage adsorption. At time zero, actively motile bacteria were mixed with phage at P/B of 0.4 in TY broth and incubated at 37 C with vigorous aeration. At 5-min intervals, 0.5-ml samples were removed and diluted with 4.5 ml of cold salts solution. The cells were immediately deflagellated; 0.05 ml of anti-PBS1 antiserum was added and, after dilution, the number of infected centers was determined. Infection was relatively rapid, being complete within 10 min (Table 1). Similar experiments in which samples were taken at 1-min intervals showed that infected centers were not formed prior to 4 min after mixing.

Effect of cyanide on adsorption and infection. KCN at a final concentration of 10^{-3} M causes immediate cessation of motility of *B. subtilis*. Table 2 shows the results of adsorption of PBS1 by SB19 rendered nonmotile by 10^{-3} M KCN. Adsorption was measured by the chloroform method. It appears that cells rendered nonmotile by KCN do not adsorb phage. If adsorption was measured by centrifuging the cyanide-treated cell-phage complex, however, only 10% of the input phage remained in the supernatant fluid (Table 3). No reduction in phage titer was found if *E. coli* or killed SB19 were substituted for the motile SB19 cells. These data suggest

TABLE 1. *Kinetics of infection of SB19 by PBS1*[a]

Time	Infected bacterial centers/ml
min	
0	$<1 \times 10^5$
5	1.2×10^8
10	2.3×10^8
15	2.1×10^8
20	2.1×10^8

[a] To 5×10^8 cells (per ml) of SB19 in TY broth at 37 C was added PBS1 to give a final concentration of 2×10^8/ml. At 5-min intervals, 0.5-ml samples were diluted into 4.5 ml of salts solution, and the bacteria were deflagellated. Anti-PBS1 antiserum was added to the samples, the mixtures were then diluted, and the number of infected centers were determined.

TABLE 2. *Effect of KCN on the adsorption of PBS1 by SB19 measured by the $CHCl_3$ method*[a]

Sample	Unadsorbed phage
Phage control	8.4×10^7
Cell control (no CN^-)	2.0×10^5
Cells + CN^-	8.1×10^7

[a] To 1.0×10^8 cells (per ml) of SB19 in TY broth at 37 C was added KCN to 10^{-3} M; the culture was shaken for 3 min in a stoppered tube. PBS1 was added to give a P/B of 1, and the mixture was shaken for 15 min more at 37 C. The numbers of unadsorbed phages were then determined.

TABLE 3. *Effect of KCN on the adsorption of PBS1 by SB19 measured by the centrifugation method*[a]

Sample	Unadsorbed phages
Phage control	8.6×10^7
Cell control (no CN^-)	1.0×10^6
Cells + CN^-	7.0×10^6

[a] See Table 2 for details. Adsorption was measured by centrifuging the bacteria-phage mixture at 3,600 × *g* for 15 min and assaying the supernatant fluid for free phages.

that chloroform separates any phage-bacteria complexes formed under KCN. To test this possibility, both the centrifugation and chloroform methods of measuring adsorption were used in the same experiment. Actively motile bacteria were incubated for 15 min with phages (P/B = 1); chloroform was added to one sample and free phage was measured. The other sample was centrifuged; the supernatant fraction was assayed for free phage, and the sediment was resuspended in the initial volume of salts solution containing 10^{-3} M cyanide and anti-PBS1 antiserum. One-half of the volume of the resuspended sediment was deflagellated and both parts of this sample were diluted and plated for infected centers. The data (Table 4) show that some adsorption takes place in the presence of cyanide, as evidenced by the facts that there was a reduction in the number of free phage in the supernatant fraction and that infected centers were formed by the sedimented cells. The data also suggest that infection does not occur in the presence of cyanide, since the sedimented cells did not form infected centers after deflagellation.

Adsorption site for PBS1. The structure of phage PBS1 was described in detail by Eiserling (6). A unique feature of this phage is the three helical tail fibers with a pitch of 350 A. After phage adsorption, these helical tail fibers are found wrapped around the bacterial flagellum (Fig. 2 and 3). There does not appear to be a single specific site on the flagellum requisite for attachment, since at high P/B the entire length of the flagellum can be covered with phages (Fig. 3 and 4).

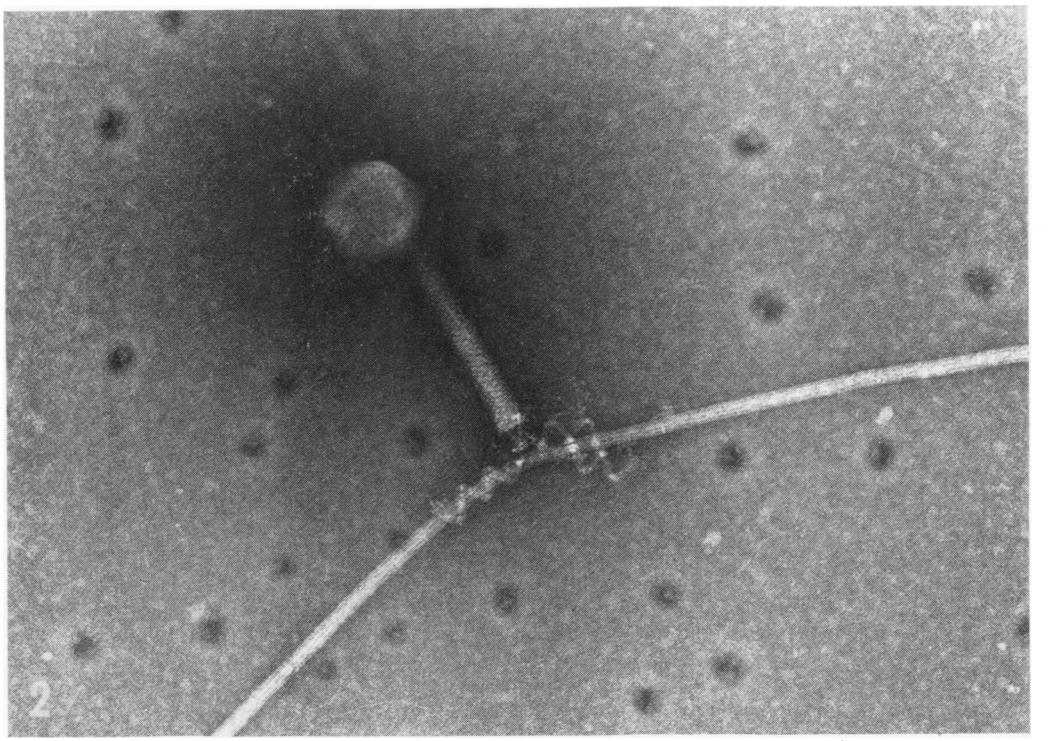
To examine further the requirement for flagella in the adsorption process, actively motile cells, deflagellated cells, and deflagellated cells whose flagella had been permitted to regenerate were tested for their ability to adsorb PBS1. Freshly deflagellated cells did not adsorb the phage to any extent, whereas bacteria whose flagella had regenerated did adsorb the phage (Table 5). Isolated flagella, either intact or prepared by shearing, did not reduce phage titer, nor did they compete with motile cells in the adsorption process. Occasionally, we observed phages attached to isolated flagella, but never a reduction in phage titer, suggesting that, if adsorption to isolated flagella does occur, it is reversible.

It was of interest to determine whether the relative length of the flagella was a critical factor in the adsorption and infection processes. Samples of a deflagellated culture of SB19 were dispensed into tubes and incubated with aeration at 37 C in TY broth. Under these conditions, *B. subtilis* will regenerate flagella maximally within 20 to 25 min. At timed intervals, phage was added to duplicate tubes (P/B = 1) and incubation was continued for 5 min more to permit adsorption. One of the duplicate tubes was treated with chloroform, and free phage was determined; the other sample was again deflagellated, anti-PBS1 antiserum was added to remove unadsorbed phage, and the number of infected centers in this sample was determined (Table 6). It appears that the adsorption process

TABLE 4. *Effect of KCN on the adsorption of PBS1 by SB19 measured by a combination of the $CHCl_3$ and centrifugation methods*[a]

Sample (supernatant)	Unadsorbed phages
Phage control	8.4×10^7
Cell control (no CN^-)	1.1×10^6
Cells + CN^-	5.3×10^7
Sample (sediment)	**Infected bacterial centers**
Cell control (no CN^-)	$>1.0 \times 10^8$
Cells + CN^-	3.7×10^7
Deflagellated cells + CN^-	$<1.0 \times 10^5$

[a] See Table 2 for details. Centrifugation of phages in the presence of *E. coli* or killed SB19 did not reduce the titer of phages from the supernatant fluid.



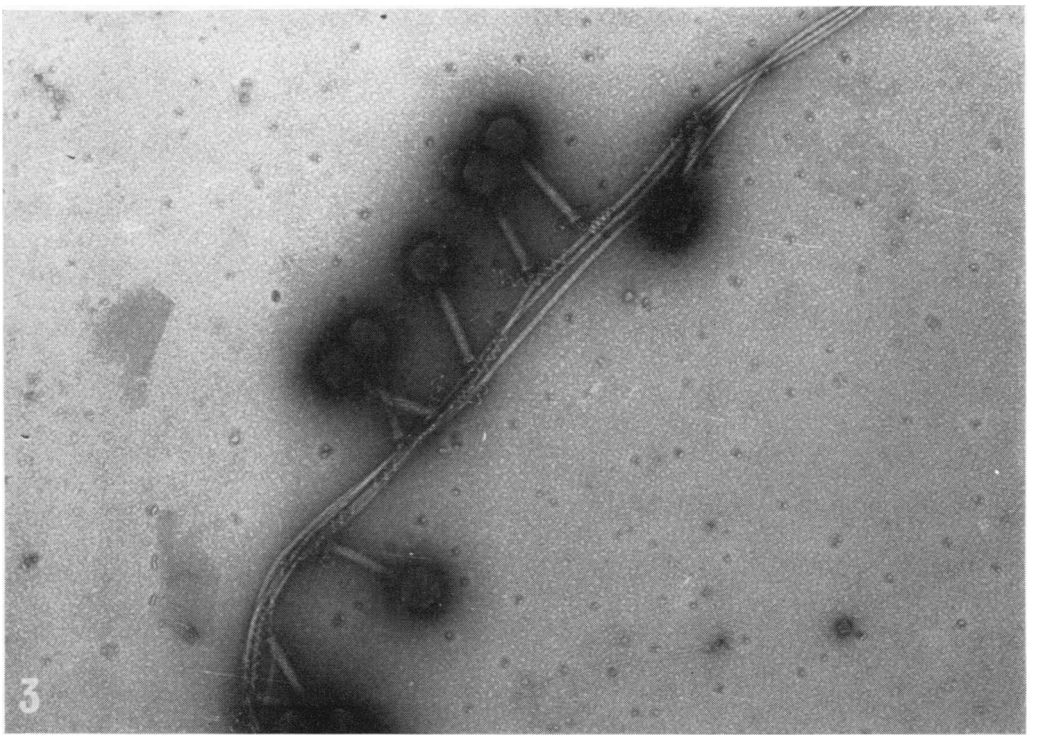


FIG. 2. *PBS1 adsorbed to the flagella of Bacillus subtilis SB19. Negatively stained with 2% uranyl acetate.* × *120,000.*

FIG. 3. *PBS1 adsorbed to Bacillus subtilis SB19. Negatively stained with 2% uranyl acetate.* × *61,000.*

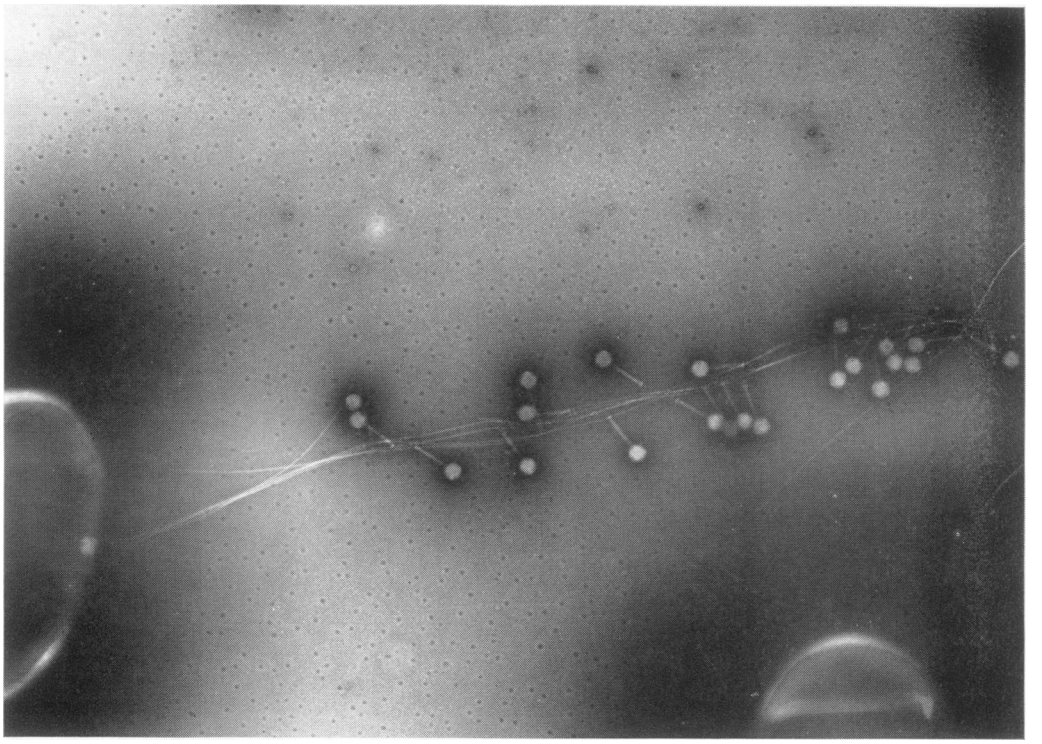


FIG. 4. *PBS1 adsorbed to Bacillus subtilis SC6. Negatively stained with 2% uranyl acetate.* × *22,000.*

TABLE 5. *Adsorption of PBS1 to deflagellated cells and cells with regenerated flagella*[a]

Sample	Unadsorbed phages
Control cells	2.0×10^4
Deflagellated cells	6.8×10^6
Cells with regenerated flagella	2.0×10^4
Isolated flagella	8.0×10^6
Phage control	8.4×10^6

[a] To 10^7 cells (per ml) of SB19 which had been freshly deflagellated, cells whose flagella had regenerated, control cells, and isolated flagella was added PBS1 to a P/B of 1. The mixtures were incubated for 15 min at 37 C in TY broth, and unadsorbed phages were measured by the $CHCl_3$ method.

TABLE 6. *Effect of relative flagella length on adsorption and infection of SB19 by PBS1*[a]

Time for flagella regeneration	Unadsorbed phages	Infected bacterial centers
min		
0	4.2×10^7	$<1.0 \times 10^5$
10	4.4×10^7	7.7×10^6
15	2.0×10^7	4.8×10^6
20	9.6×10^6	1.2×10^7
30	8.0×10^6	1.5×10^7
50	3.0×10^6	1.5×10^7

[a] SB19 cells were deflagellated, washed once with TY broth, suspended in TY broth in separate tubes, and incubated at 37 C with aeration. At time intervals, phage was added to duplicate tubes (P/B = 1) and incubation was continued for 5 min more. One of the tubes was assayed for adsorption by the $CHCl_3$ method, and the other tube was deflagellated again, anti-PBS1 antiserum was added, and the number of infected bacterial centers was determined.

is more rapid as the relative length of the flagellum increases, i.e., the longer the time permitted for flagella regeneration, the greater the reduction in free phage.

The complexing of specific antiflagellar antibody to the surface of flagella results in immobilization of the bacteria. We determined whether flagella coated with antibody could act as receptors for PBS1. A culture of SB19 was diluted to contain approximately 10^7 cells/ml so that, upon addition of antiflagellar antibody, the cells would not agglutinate. After mixing bacteria with purified antiflagellar antibody for 15 min at

37 C, phage was added at a P/B ratio of 0.6 to 1.0 and incubation was continued. Samples were taken promptly at the time of addition of the phage and after 15 min of incubation. Phage adsorption was measured by the chloroform method. No adsorption of phage was detected when flagella were first complexed with their specific antibody (Table 7). The purified antibody preparation did not exhibit any neutralizing effect on the phage, since no reduction in phage titer was observed when free phage were treated with antibody under the same conditions.

It has been demonstrated by Weilbull (17) that protoplasts of flagellated bacilli retain their flagella yet are nonmotile. We measured the adsorption of PBS1 by protoplasts of SB19 to determine whether the cell wall was involved in the adsorption process. The protoplast preparations in 20% sucrose were incubated with phage at P/B of 0.2 on a reciprocal shaker, and adsorption was measured by the chloroform technique. To determine whether the protoplast preparations retained significant amounts of cell wall, the adsorption of phage SP8 was also measured. Phage SP8 is known to adsorb to the cell wall of *B. subtilis* (W. R. Romig, *personal communication*).It is evident (Table 8) that PBS1 was adsorbed by the protoplasts, whereas there was no reduction in the titer of SP8. Although incubation was carried out for 60 min, no bursts were detected in any of the experiments. However, one cannot conclude that infection and phage maturation did not occur. Bursts obtained from protoplasts may be as low as 5% of the normal (3), so that a similar reduction of the burst for PBS1 might not be detectable. Alternatively, infection and phage maturation may not have taken place in the protoplast preparations. Further, under the conditions employed there was a reduction in the optical density of the protoplast preparations, suggesting that some lysis was occurring.

TABLE 7. *Effect of specific antiflagellar antibody on PBS1 adsorption*[a]

Sample	Time	Unadsorbed phages
	min	
Control (no antibody)	0	5.0×10^6
	15	1.5×10^6
Cells treated with antiflagellar antibody	0	6.7×10^6
	15	6.9×10^6

[a] SB19 cells (5×10^6/ml) were treated with specific antiflagellar antibody for 15 min at 37 C; phage was then added (P/B = 1.2) and incubation was continued for 15 min more. Unadsorbed phages were measured by the $CHCl_3$ method.

TABLE 8. *Adsorption of PBS1 by protoplast preparations of SB19*[a]

Time	Unadsorbed phages	
	PBS1	SP8
min		
0	1.7×10^8	3.8×10^7
1	1.4×10^8	
5	6.9×10^7	3.6×10^7
15	2.9×10^7	4.4×10^7
30	9.8×10^6	4.0×10^7
45	5.0×10^3	

[a] Protoplast preparations of SB19 in TY broth containing 20% sucrose were mixed with PBS1 (P/B = 0.2) at 37 C in a reciprocal shaker. At time intervals, samples were assayed by the $CHCl_3$ method for free phage. Phage SP8 was used as control. Phage SP8 was found to adsorb normally to vegetative cells in 20% sucrose.

The possibility was considered that interaction between the helical tail fibers of the phage and the flagellum might alter the attachment mechanism of the phage. Attachment to the cell wall of bacteria could conceivably then follow. To test this possibility, 5×10^7 SB19 cells were incubated for 5 min with phage at P/B of 40, at which time 5×10^8 cells of SB171 (*fla*$^-$) were added and incubation was continued for 10 min more. A control tube did not receive the SB171 cells. Anti-PBS1 antiserum was then added, and the number of infected centers was determined. If the attachment mechanism of PBS1 were modified by reaction with the flagellum, we would anticipate that the number of infected centers would be greater in the tubes having SB171 cells, owing to transfer of modified phages from SB19 flagella. The numbers of infected centers obtained in the tubes having SB171 (30.3×10^6) were essentially the same as those in the control tubes (36.9×10^6).

Frankel and Joys (7) reported that four nonflagellated mutants of *B. subtilis* would not adsorb PBS1. We extended this observation with >50 *fla*$^-$ mutants of 168, and confirmed that none adsorbed the phage. Frankel and Joys (7) also reported that a paralyzed mutant of *B. subtilis* did not adsorb PBS1, and concluded from this observation that functional flagella were required for PBS1 adsorption. However, we found that PBS1 adsorption does occur with four mutants of 168 which are nonmotile because the flagella formed by these cells lack the long period helix (Fig. 4). Table 9 gives the data obtained for adsorption of PBS1 by mutant SC4. The mutants lacking the long period helix not only adsorb the phage but are also susceptible to phage infection

(Table 10). Thus, the capacity of flagella to function for motility may be lost without loss of their capacity to adsorb phage and permit infection.

We tested the effect of adsorption of PBS1 by SB19 on the motility of the cells. Meynell, using *chi* phage (11), and Frankel and Joys, using PBS1 (7), reported the cessation of motility of the bacteria when mixed with phage at high P/B. Both of the phage preparations employed by these workers were crude lysates. Phages purified by banding in CsCl were used in our experiments at various P/B ratios. The cultures of SB19 used showed more than 95% motile cells. Cells were mixed with phage, and the fraction of the cells remaining motile was estimated by examination in a phase microscope. The time interval between mixing bacteria and phage and scoring for motility was about 15 to 30 sec. At P/B of 1, there were approximately 50% motile cells; at P/B of 2.5, there were 10 to 25% motile cells; at P/B of 5, no motile cells were observed. These numbers agree fairly well with the Poisson distribution for phage adsorption, suggesting that one plaque-forming unit when attached to one flagellum on the bacterial cell induces a rapid cessation of motility.

TABLE 9. *Adsorption of PBS1 by Bacillus subtilis SC4*[a]

Sample	Unadsorbed phages
SB 19	$<1.0 \times 10^5$
SC 4	6.0×10^6
Phage control	1.2×10^8

[a] Strains SB19 and SC4 were mixed with phage (P/B = 0.1) for 20 min at 37 C with vigorous aeration. Phage adsorption was measured by the $CHCl_3$ method.

TABLE 10. *One-step growth of PBS1 on Bacillus subtilis SC6*[a]

Time	Infected bacterial centers
min	
15	1.9×10^6
20	2.2×10^6
25	2.1×10^6
30	2.3×10^6
35	2.0×10^6
40	2.3×10^6
45	3.2×10^6
50	4.5×10^6
55	1.2×10^7
60	1.6×10^7
70	3.0×10^7

[a] Strain SC6 was mixed with phage (P/B = 1) in TY broth at 37 C with vigorous aeration. At time intervals, samples of the culture were removed and one-step growth characteristics were measured by the method of Adams (2).

DISCUSSION

The data presented in this communication clearly demonstrate that phage PBS1 attaches to the flagella of *B. subtilis*. These data may be summarized as follows.

In electron micrographs, the helical tail fibers of the phage are clearly seen, wrapped around the flagella. Rarely, if ever, are the phages seen attached to the cell body, especially if P/B ratios of less than 10 are used.

The *fla*$^-$ mutants do not adsorb the phage. This in itself would not be compelling evidence that flagella are involved in phage adsorption. It is possible that *fla*$^-$ mutants may have altered cell envelopes, perhaps due to a secondary mutation or because the absence of flagella modifies the conformation of the envelope.

Deflagellated cells do not adsorb PBS1, nor are they susceptible to phage infection. This is more direct evidence for the involvement of flagella in the adsorption process, especially when coupled with the observation that the same culture, when permitted to regenerate flagella, becomes susceptible to phage infection.

When the surface of flagella is coated with specific antibody, phage adsorption is completely inhibited. This evidence might suggest that functional flagella are required for phage adsorption. This interpretation appears highly unlikely when one considers that paralyzed mutants, as well as cells rendered nonmotile by treatment with cyanide, adsorb phage with essentially the same kinetics as do normal cells. Alternative explanations which appear more tenable are that coating the flagellum with antibody increases the functional diameter of the flagellum, thus preventing the helical tail fibers of the phage from attaching, or that attachment sites on the flagellum are coated with antibody, thus hindering the adsorption mechanism of the phage.

Protoplasts of *B. subtilis* retain the ability to adsorb PBS1, yet are not susceptible to phage SP8. The rate of adsorption of PBS1 by the protoplasts is slightly slower than that observed with vegetative cells. This may be ascribed to the fact that some of the flagella are lost in the process of preparing the protoplasts and, further, the incubation of the phages with protoplasts was carried out under suboptimal adsorption conditions, i.e., gentle shaking in 20% sucrose rather than under vigorous aeration. Nevertheless, protoplasts are nonmotile and are devoid of cell wall. This suggests that cell wall material, as such, does not play an essential part in the adsorption

process. It also lends further support to the observation that nonfunctional flagella can serve as attachment sites for PBS1.

These data, then, lend credence to the conclusion that it is on the flagellum that initial contact is made between PBS1 and the bacterium. The data also demonstrate that functional flagella are not prerequisite for phage adsorption, since cells rendered nonmotile by cyanide, by production of nonfunctional flagella, or by protoplasting all served as suitable adsorbers of the phage. This is contrary to the report by Frankel and Joys (7) and the observations of Meynell (11) and Schade and Adler (13), who proposed that functional flagella were requisite for phage adsorption. Further studies on the *chi* phage by Schade, Adler, and Ris (14) revealed that this phage also adsorbs to nonfunctional flagella.

The time required for infection of *B. subtilis* by PBS1 appears to be considerably longer than *Salmonella* infection by phage *chi* (11). No infected bacteria were observed prior to 4 min (Table 1) after mixing PBS1 with SB19, and yet Meynell (11) reported that *chi* infection of *Salmonella* takes place within 1 min after mixing. The time discrepancy may be due to the relatively large amount of DNA that must be transferred by PBS1 in comparison to the DNA in *chi* (2×10^8 versus 4×10^6 daltons, respectively).

It is tempting to propose from the data presented in Table 4 that DNA injection by PBS1 may require cellular energy. It is clear that injection is inhibited by cyanide, since the cyanide-treated bacteria did not become infected if they were deflagellated prior to plating. Further, the cyanide experiments also suggest that there may be two steps to the adsorption process: a cyanide-insensitive reversible step, after which infective phage may still be removed by a chloroform treatment, followed by an irreversible step that is prevented by cyanide. It would appear that the reversibility of the initial adsorption step is not merely due to lack of translational motility in the cyanide-treated culture, since phage adsorption onto other nonfunctional flagella (protoplasts, paralyzed mutants) was measured by the chloroform method and was not found to be reversible.

A prime question which has not been resolved by these studies is the route by which PBS1 DNA enters the bacterial body. There are several mechanisms which may be considered. (i) Direct injection of PBS1 DNA into the flagellum is one possibility. If we consider, however, that a central canal has not been observed in the flagella of *B. subtilis* (although such a canal might indeed exist), and that phages with contracted sheaths and empty heads are rarely seen attached to the flagella, then this proposal seems unlikely. Further, one would expect that injection of DNA into the flagellum might result in a morphological alteration of the flagellum at the site of injection, and no such changes have been observed. (ii) That DNA injection does not occur via a modified transfection mechanism (12) after phage adsorption was ruled out by the fact that the infectious process is completely deoxyribonuclease- resistant (W. B. Pritikin, Ph.D. Thesis, Univ. of California, Los Angeles, 1967). (iii) The fact that nonflagellated cells do not become susceptible to phage infection also suggests that initial adsorption to the flagellum does not alter the attachment mechanism so that a secondary adsorption to cell walls can follow. This experiment does not rule out the possibility that initial attachment to the flagellum of one cell may be followed by transfer of the phage to the wall of the same cell. Indeed, this is in essence the mechanism proposed by Schade, Adler, and Ris (14) for *chi* phage DNA transfer. These workers suggested that *chi* phage attaches to the flagella of *E. coli* and then slides along the flagellum to the base, where DNA injection occurs. They suggested that the movement of the flagellum is responsible for, or aids, in the sliding of the phage down the length of the flagellum, although they presented evidence demonstrating adsorption to nonfunctional flagella. Although we have no evidence which supports this hypothesis, it is the only tenable one at present. We would, however, expect that the shorter the flagellum, the more rapid the infection, since the phage would have a shorter distance to travel from the site of attachment. This does not appear to be the case (Table 6). Further, there are arguments which may be invoked against such a mechanism. A moving body produces a column of fluid which moves in a direction opposite to that of the direction of motion of the cell. This moving column of fluid would create an impedance against the sliding of the phage particle toward the cell body. However, it is also known that there exists a fixed, nonmoving boundary of fluid which surrounds moving objects. Although the functional area covered by such a boundary has not been clearly measured for bacterial systems, it may be sufficiently large to eliminate the argument of impedance posed above. Thus, the question still remains: how does PBS1 infect *B. subtilis*?

Finally, the phenomenon of immobilization of *B. subtilis* by CsCl-purified phages merits discussion. It appears from the data that adsorption of one phage particle to one flagellum of *B. subtilis* renders the entire complement of flagella nonfunctional. This is a rather dramatic observation, especially when one considers that this bacterium possesses 15 to 20 flagella and that the

time required to immobilize the cell is less than 30 sec. Although infected bacterial centers do not appear before 4 min after addition of phage, we considered the possibility that the cessation of motility might be due to the initiation of DNA injection with some concomitant channeling of energy. To test this possibility, phage ghosts were prepared by osmotically shocking PBS1 by rapid dilution from 35% glucose. The deoxyribonuclease-treated ghosts immobilized the bacterial cells as effectively as did intact phages. The fact that the entire complement of flagella is immobilized suggests that the functioning of all the flagella of *B. subtilis* is under the control of one "motor."

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