Role of F Pili in the Penetration of Bacteriophage f1

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Early stages of infection of *Escherichia coli* with the filamentous bacteriophage f1 were examined in the electron microscope. Purified phage-bacteria complexes were prepared at various time intervals after the initiation of synchronous infection. Cells were scored for the total number of F pili, the number of F pili with f1 attached, the number of intact phage particles which occurred at the surface of the cell, and F pilus length. Electron microscope autoradiographs were also prepared at each time interval. The results showed that the average number of F pili with f1 attached decreased with time as phage deoxyribonucleic acid (DNA) entered the cell. Concomitant with this loss, the remaining F pili became shorter. The rate of entry of phage DNA into the cell followed, with a short lag, the rate of loss of F pili with f1 attached. During the lag period, intact phage particles accumulated at the surface of the cell. The results from radioautographs showed that no phage DNA could be located within the F pilus. These results suggest that F pili are resorbed by the cell during infection with the bacteriophage f1. Parallel experiments with noninfected cultures further suggest that pilus resorption may be a normal cellular phenomenon.

The filamentous bacteriophage f1 is one of a class of immunologically related bacteriophages which only infect bacterial cells carrying a fertility factor (13, 17). It was first described by Loeb in 1960 (11) and further characterized by Loeb and Zinder (12). The phage particle is approximately 800 nm long, 5.5 nm in diameter, and contains a single-stranded circular molecule of deoxyribonucleic acid (DNA) with a molecular weight of 2×10^6 (13, 17). Its cellular attachment site is the tip of the F pilus (5). Phage attachment is an irreversible process which occurs at 0°C as well as at higher temperatures. Measurable penetration of phage DNA into the cell only occurs at elevated temperatures (19). Little else is known concerning the initial steps in the infection process. Several years ago, Trenkner et al. (18) showed that phage coat protein, as well as phage DNA, entered the host cell during the course of infection with the bacteriophage fd. Since an entire phage particle would be too thick to enter the F pilus, Marvin has proposed that the F pilus is retracted by the host cell as a first step in infection (13). In support of this model, he showed that F pili were no longer visible on cells which were infected with phage and that the disappearance of F pili (assayed by the binding of radioactive ribonucleic acid phage) paralleled the production of cells infected with fd. These observations, however, were apparently made at late times after phage infection, and it has therefore

been suggested that they may result from the inhibition of new F pili synthesis after infection is established within the cell (4). In addition, Henry has recently restudied the fate of coat protein after infection with the bacteriophages fd and M13 and shown that although phage protein can be found within the cell after phage infection its presence is not essential for successful infection and probably occurs as a secondary event (8).

In an alternate model, Brinton has proposed that F pili function as a conduit for the transport of phage nucleic acid (2, 3). In Brinton's model, the F pilus also serves to transport DNA from the donor into the recipient cell during bacterial conjugation and thus is capable of transporting nucleic acid molecules in two directions. Infection of donor cells with male-specific bacteriophages either prior to or during bacterial conjugation inhibits the transfer of DNA into the recipient cell (9, 10, 15, 20), suggesting that phage infection and the transfer of DNA during bacterial conjugation may well occur by an identical mechanism.

In this paper, we present some additional information concerning the mode of entry of the filamentous phage f1 into the bacterial cell. We have looked for the presence of phage DNA within the F pilus by electron microscope autoradiography at various times after the initiation of infection. In addition, we studied the changes in distribution of F pili, with and without attached

phage, as well as the alteration of median length of F pili as the infection process proceeds. Our data support a model in which the F pilus is withdrawn into the host cell as a first step in the infection process.

MATERIALS AND METHODS

Media and bacterial strains. The following media were used: L broth (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.1% glucose, pH 7.0), maltose-tryptone broth (1% tryptone, 0.5% NaCl, 0.4% maltose), and M-9 (0.7% Na_2HPO_4 , 0.3% KH_2PO_4 , 0.05% NaCl, 0.1% NH_4Cl , 0.4% glucose, 10^{-4}M CaCl_2 , 10^{-3}M MgSO_4 , thymine [2 $\mu\text{g/ml}$], vitamin B1 [200 $\mu\text{g/ml}$]).

The bacterial strains were obtained from L Caro. The Hfr strain of *E. coli* K-12 (Hfr OR11 *leu⁻thy⁻*) (1) was used both to prepare phage stocks and for all the experiments described. An isogenic F⁻ strain derived from the above strain was used as a control.

Phage stocks. Labeled f1 was prepared and purified as follows. Cells were grown in 5 ml of M-9 medium containing ^3H -thymine (12–19 Ci/mole, New England Nuclear) for 2 hr ($\sim 5 \times 10^7$ cells/ml). They were then infected with a total of 2×10^8 f1, and the culture was allowed to grow overnight to a final titer of 2×10^{11} to 4×10^{11} phage per ml. The culture was centrifuged to remove cells and the supernatant fluid was digested with deoxyribonuclease (60 $\mu\text{g/ml}$) and then dialyzed four times against 1 liter of a solution containing 0.1 M tris(hydroxymethyl)aminomethane (Tris) and 0.1 M NaCl, pH 7.0. The dialysate was adjusted to pH 4 with acetic acid, after which 0.2 volume of 95% ethanol was added. After several hours at 6 C the floc which formed was collected by centrifugation; the pellet was dissolved in the Tris-NaCl solution, adjusted to a final density of 1.3 with cesium chloride, and centrifuged to equilibrium for 24 hr at 39,000 rev/min in an IEC-B60 centrifuge using the SB405 rotor. Such stocks contained virtually no acid-soluble counts and gave a single homogeneous peak when sedimented in 5 to 20% sucrose gradients.

The MS2 stock was prepared and purified by the method of Loeb and Zinder (12).

Measurement of phage label inside cells. External f1, attached to F pili was removed from cells by a brief ultrasonic treatment with a Sonifier Cell Disruptor (Heat Systems Co. Melville, L.I., N.Y.) using a microtip on the probe. A 1.0-ml amount containing 2×10^8 cells was treated for 30 sec at a power setting of 1. Foam was dispersed with Dow-Corning Antifoam spray. The cells were then centrifuged, the pellet was dissolved in distilled water, and 0.1 ml of the cells was dried on filter paper discs for radioactive assay. Alternatively, the cells were digested with 1 N NaOH at 80 C for 30 min and were mixed with 2 ml of Scintisol TD and 10 ml of Scintisol Complete (Isolab Incorporated). From this latter procedure, the correction factor for the self-absorption of cells in the disc procedure was determined. Free phage or phage attached externally to the cells counted with equal efficiency on discs or by the alkaline digestion method.

Application of cells to grids for electron microscopy.

Cells which had previously been fixed in 1 to 5% Formalin at room temperature for approximately 24 hr were centrifuged and gently suspended in distilled water. At the same time, grids (400-mesh copper screen-coated with Formvar and carbon films) were treated by glow discharge. The grids were then placed on Parafilm, and a drop of cell suspension was applied to each grid. (Parafilm was used to prevent the drops from spreading and thus facilitated the handling of large numbers of grids simultaneously.) Cells were allowed to attach to the grids for 15 min, and nonattached cells were then removed by inverting the grids over cold 25% ethanol or distilled water. The grids were drained on filter paper, air dried for several hours, and shadowed with palladium-gold (40:60) wire on a rotary platform at arc tangent 0.6/11. The center of the rotating platform was located 11 cm from the filament.

EM autoradiography. Shadowed grids were coated with carbon, placed on glass slides, and coated with Ilford L4 emulsion (6). The grids were developed in Microdol X (Kodak) after 3 to 4 months of exposure.

Pili enumeration and length measurement procedures. The number of pili per cell was determined by examining cells directly on the fluorescent screen of a Siemens Elmiskop I electron microscope. For each treatment, 300 cells were scored; 100 on each of three different grids. Cells were scored only if the tips of all pili were visible, and every countable cell was scored in each grid square examined. In general, every other grid square was counted until 100 cells were scored. If a particular square was not usable, the next adjacent square was counted.

To measure F pili lengths, cells were photographed at $\times 3,600$. Again, entire grid squares were photographed by taking two to three overlapping films. Photographs were taken from three different grids, and at least 150 pili were measured in each class. For measurement, the negatives were projected to a final magnification of 37,600 and the pili lengths measured with a curvimeter.

RESULTS

Adsorption of phage to bacteria and preparation of purified phage-bacteria complexes. For quantitative electron microscopy, it was necessary to prepare purified phage-bacteria complexes in which the cells had many F pili, where efficient attachment of the phage had occurred and where phage penetration into the cell could be synchronized. Optimal piliation of the cells was achieved by growing cells in L broth with limited aeration until the end of log-phase growth. Under the conditions of restricted aeration, this occurred at a cell density of 2×10^8 per ml. Phage were attached to cells at 0 C, conditions under which no phage DNA enters the cell (19), at a multiplicity of 75 to 100. Maximal attachment of the phage particles occurred within 15 min, and in the electron microscope generally 50 to 60% of the F pili had at least one phage particle attached. Excess

free phage was then removed by centrifugation through 5% sucrose (for details see legend to Table 1) or filtration through Oxoid membrane filters. This last procedure, although efficient in removing excess phage, resulted in substantial pili breakage. Therefore, the method of centrifuging cells through sucrose was preferred. Virtually all phage particles which were not specifically bound to F pili were removed (Table 1); very little pilus breakage occurred, and approximately 90% of the cells remained viable after the treatment.

Penetration of phage DNA into the cell. The amount of f1 DNA which entered the host cell at various time intervals was determined with phage-bacteria complexes in which the phage DNA was labeled with ^3H -thymine. Chilled, purified, phage-bacteria complexes were incubated at 37 C for various time intervals to allow entry of phage DNA into the cell. External phage were removed from the cells by a brief ultrasonic treatment as detailed above. The ultrasonic treatment followed by one centrifugation step was sufficient to remove all label on the outside of the cells (Table 1); the cells also appeared free from F pili in the electron microscope. Cell counts performed with the light microscope showed no evidence of lysis due to sonic treatment. Most phage penetration occurred within 5 min of the transfer to 37 C, and no further penetration occurred after 10 min. In general, the rate of penetration was linear for the first 3 or 4 min and reached a plateau by min 6 (see Fig. 3). Often, however, a brief lag followed by a second increase in the rate of penetration was observed somewhere between

min 2 and 4. Both the occurrence and position of this lag varied in different experiments, and its significance was not determined.

Autoradiography of phage-bacteria complexes. In parallel to the penetration assays described above, phage-bacteria complexes were prepared for electron microscope autoradiography. In this instance, the penetration of phage DNA into the cell was interrupted at various intervals by the addition of 5% Formalin. All samples remained in the 37 C water bath for a total of 10 min. The samples were then chilled and divided in half. One half was used to measure the amount of phage DNA which had entered the cells, and the other half was used to prepare radioautographs. Penetration curves after the Formalin treatment were identical with those obtained by immediate ultrasonic treatment.

Figure 1 is an electron micrograph typical of the sort obtained in these experiments. Silver grains are readily visible over phages at the tips of F pili. The black area over the cell is an artifact due to the interaction of the cell with the emulsion. F pili are clearly distinct, and if silver grains had occurred over these structures they would have been readily visible.

In all, eight separate experiments were prepared and examined. Significant numbers of silver grains were never found associated with F pili although many grains were always present over phage. In one experiment, 100 cells were scored at each time interval for the presence of grains over pili. In the first 4 min of the experiment, two grains were observed over a total of 204 F pili. Of these same pili, 52 had phage attached, and the average number of grains over phage particles was 1.7 (88 grains). The two grains over the F pili could easily have been due to background.

Disappearance of F pili in the presence of f1. A surprising observation was made during the autoradiographic experiments. F pili, particularly those with f1 attached at the tip, seemed to disappear with time as phage DNA entered the cell. To quantitate these observations, three experiments were performed in which the number and length of F pili, as well as the amount of phage DNA which entered the cell at different time intervals, were measured. Special precautions were taken to minimize pilus breakage during the preparation of phage-bacteria complexes and during the subsequent steps for the preparation of the grids. To exclude the possibility that preferential binding of a particular class of cells to the grids might occur, reconstruction experiments were performed with known mixtures of Hfr and F⁻ cells. These experiments showed that the relative proportion of each cell type which was deposited on the

TABLE 1. *Purification of phage-bacteria complexes*

Treatment	Radioactivity (counts/min)
Initial Mixture	
Hfr + f1- ^3H	70,000
F ⁻ + f1- ^3H	69,000
After sucrose purification	
Hfr + f1- ^3H	1,200
F ⁻ + f1- ^3H	150
After sonic treatment	
Hfr + f1- ^3H	150

* Phage bacteria complexes were prepared as described in the text. After 15 min at 0 C the non-attached phage were removed by carefully layering the cells over 5% sucrose in tryptone broth and then centrifuging them at $4,000 \times g$ in a swinging-bucket rotor for 5 min. The supernatant fluid was discarded, and the pellet was gently suspended into broth. The preparation was then sonically treated and centrifuged again. The supernatant fluid was discarded, and the pellet was resuspended and assayed.

FIG. 1. Electron microscope autoradiograph of a cell of *Escherichia coli*, incubated for 2 min at 37 C, showing the distribution of silver grains over phage particles. F pili are identified by the attachment of MS2 along their length. The variation in phage length which can be observed is due to the presence of longer f1 particles in the infecting phage stock. Such particles, when measured, are always multiples of the unit phage length. Magnification: $\times 15,200$.

grids was directly proportional to its concentration in the mixture from which the grids were prepared and, therefore, that cell binding to the grids was not affected by the presence or absence of F pili.

The results obtained in these experiments confirmed our preliminary observations; F pili became shorter and finally disappeared as phage DNA entered the cell. The distribution of cells shifted from an initial population in which most cells had several F pili to a population where many cells had none. In particular, almost all

pili with f1 attached disappeared. The data of one experiment are presented below.

Figure 2A shows the distribution of cells according to the number of F pili protruding from their surface. At the beginning of the experiment, most of the cells had several F pili, occasionally as many as 12. With time, as phage DNA entered the cells, the distribution changed. In the first minutes, the relative proportion of cells with more than three F pili decreased, while the number of cells with one and two F pili increased. By min 3, the number of cells in these

FIG. 2. Changes in the distribution of F pili on cells with time. A, Total F pili per cell; B, F pili with f1 attached per cell.

classes also dropped and an increase was only observed in the 0 class. After 5 min, cells with five and six pili appeared again. The reappearance of these cells corresponded to a net increase in the total number of F pili in the population and seems to correspond to the net synthesis of new F pili.

Since, at the beginning of the experiment, 55% of the F pili present on cells had phage attached to their tip, it is of interest to examine the distribution of cells specifically with regard to these pili. These data are presented in Figure 2B. At the beginning of the experiment, 77% of the

cells had phage attached to at least one pilus, and some cells had as many as eight F pili with phage attached. With time, almost all of these pili disappeared from the cells.

These data are summarized in Table 2, which provides information on the mean number of F pili per cell with and without f1 attached. It can be seen that at the beginning of the experiment there were an average of 3.39 F pili per cell. Their number decreased to 0.73 by min 4. There were an average of 1.86 pili with f1 attached at the beginning of the experiment, and their value decreased to 0.14. F pili without f1 attached also disappeared from the cells. In general, this loss occurred during the first minute of the experiment, and subsequently the number of pili which remained on the cells was fairly constant. However, further losses of these pili could have occurred and be balanced by new growth of pili. This would not have been detected since the pili were not labeled in any way and therefore could not be distinguished from pili which were newly synthesized. The additional loss of F pili without f1 attached which occurred in min 4 is probably an artifact since it was not seen in other experiments.

Data on the median length of F pili can be seen in Table 3. In the first 3 min of the experiment, the median length of F pili with f1 attached dropped from 2.3 to 1.2 μm . Data were not collected beyond this time primarily because the number of F pili with f1 attached which remained in the preparation was so low that it would have required a very large number of photographs to obtain reliable measurements. Furthermore, in min 4 some cells were observed which still had several long pili with phage attached. Presumably these cells represent non-viable cells produced during the purification of phage-bacteria complexes. Such cells are only a small proportion of the total population, but the presence of a few cells with very long F pili introduces a significant bias into the length determinations when most of the F pili on viable cells have disappeared.

The entry of phage DNA versus the loss of F pili. When the loss of F pili with f1 attached was compared with the entry of f1 DNA into the cell (Fig. 3), it was seen that F pili with f1 attached disappeared faster than f1 DNA entered the cell. Although this observation was initially disconcerting, it soon became apparent that the lag in the entry of phage DNA into the cell corresponded to a transient accumulation of phage particles at the surface of the cell. Since the filamentous phages are only slightly longer than the average diameter of the normal *E. coli* cell,

TABLE 2. *The loss of F pili during infection with the bacteriophage f1*

Time (min)	No. of F pili per cell	No. of F pili +f1 per cell	F pili remaining (%)			f1 at surface of cell (per cell)
			With f1	Without f1	Total	
0	3.39	1.86	100	100	100	0.24
1	2.41	1.33	72	71	71	0.39
2	1.52	0.58	31	61	45	0.68
3	1.47	0.37	20	72	43	0.65
4 ^a	0.73	0.14	8	39	22	0.43
5	1.35	0.17	9			0.58
6	1.79	0.23	12			0.44
10	1.06	0.14	8			0.25

^a The data for the 4-min time interval are probably not accurate since the grids were of poor quality, i.e., the cells were clumped, and many broken pili were visible.

TABLE 3. *The change in median length of F pili during infection with the bacteriophage f1*

Time (min)	Median length (μm)	No. of pili per cell	Total length of pili per cell (μm)	Pili remaining (%)
F pili with f1 attached				
0	2.3	1.86	4.2	100
1	1.7	1.33	2.2	52
2	1.3	0.58	0.77	18
3	1.2	0.37	0.44	10
F pili without f1 attached				
0	2.4	1.61	3.8	100
1	2.1	1.12	2.4	63
2	1.5	0.98	1.4	37
3	1.3	1.12	1.5	39

phage particles protruding directly from the cell surface were difficult to score accurately. Nevertheless, the accumulation of phage on the cell surface in min 2 and 3 of the experiment was clear (last column of Table 2).

The accumulation of phage at the surface of the cell is also illustrated in two of the electron micrographs of Fig. 4. Figure 4A shows a group of cells at the beginning of the experiment. In Fig. 4B and C, the cells were incubated at 37 C for 3 min. In Fig. 4B, F pili are no longer visible on the cell, but six phage particles can be seen extending from the surface. A few short F pili can be seen in Fig. 4C. Phage are not attached at the tips of these pili but do appear at the surface of the cell.

Loss of F pili in the absence of f1. To determine whether F pilus loss occurs in the absence of f1, cells were prepared for electron microscopy in the absence of f1, but otherwise under conditions identical with previous experiments. They were

FIG. 3. *Comparison of the entry of phage DNA into the cell with the loss of F pili that have f1 attached as a function of time. Symbols: ○, percent total phage DNA that enters the cell; △, percent total F pili with f1 attached that are lost.*

chilled at 0 C for 15 min, centrifuged through 5% sucrose, resuspended in broth, and then incubated at 37 C. The results obtained are shown in Table 4. After 4 min at 37 C, only 52% of the F pili present at the beginning of the experiment remained on the cells. The median length of the pili dropped from 2.6 to 1.6 μm.

DISCUSSION

The results show that the average number of F pili with f1 attached decreases as phage DNA enters the cell. Concomitant with this loss, the remaining F pili become shorter. In addition, the increase in the amount of phage DNA which enters the cell with time follows, with a short lag, the loss of F pili. During the lag period, phage particles accumulate at the surface of the cell. Moreover, phage DNA was not detected within the F pilus by electron microscope autoradiography.

Brinton has proposed that the F pilus functions as a conduit for the transport of phage nucleic acid into the cell and has further suggested that the normal mechanism of F pilus formation entails outgrowth to a fixed length followed by

FIG. 4. Electron micrographs showing the distribution of F pili on cells. A, Cells at the beginning of the experiment. Numerous F pili extend from the surface of the cells. To identify these structures, MS2 was attached 30 to 40 min after the cells were fixed with Formalin. The arrows indicate f1 particles attached at the tips of F pili. B, Cell from min 3 of the experiment. No F pili are visible, but several f1 particles can be seen at the surface of the cell. C, Two cells taken from min 3 of the experiment. Two short F pili are visible. Several phage particles can be seen at the surface of both cells. Magnification: $\times 10,400$.

TABLE 4. *The loss of F pili in the absence of fl*

Time (min)	F pili per cell	Percent remaining	Median length (μ m)	Total length per cell	Percent remaining
0	1.64	100	2.6	4.3	100
1	1.44	88	1.9	2.7	63
2	1.26	77	1.6	2.0	47
3	1.00	61	1.6	1.6	37
4	0.85	52	1.6	1.4	33
5	1.05				
10	1.04				

subsequent detachment from the cell (2). Our results are not consistent with this model.

A simple detachment mechanism could account for the observed loss of F pili with fl attached and, if the process were length specific, for the apparent shortening of such pili. To test this, we centrifuged purified phage-bacteria complexes after incubation at 37 C for various time intervals and examined the supernatant fractions for the presence of labeled phage. After 10 min at 37 C, no significant increase was found in the number of labeled phage which could be separated from the cells by centrifugation. Thus it seems unlikely that many F pili with fl attached at the tip detach from the cells.

In addition, the conduit model does not account for the transient accumulation of phage particles at the cell surface or for the negative results of the autoradiography experiments. With respect to the former, it must be remembered that all experiments were performed with purified phage-bacteria complexes. No detectable free phage particles which might later attach at the cell surface were found in the medium. The binding of phage particles to the tip of the F pilus has been shown to be an irreversible process (19), so that it is unlikely that phage particles detach from the pilus and migrate to the cell surface.

A simple mechanism which accounts for all of the observed experimental facts is F pilus resorption. Pili with phage attached are resorbed by the cell, thus bringing phage to the surface of the cell. Clearly, these pili would become shorter and disappear with time. Phage DNA enters the cell as a second step, thus accounting for the lag in the entry of phage DNA into the cell, as well as the accumulation of phage particles at the surface of the cell. The decrease in the length of F pili to which phage are not attached may result in part from resorption and in part from the outgrowth of new F pili which would initially be short also. Since a pilus detachment model cannot be devised to account for all the experimental data, it seems likely that F pilus resorption occurs. It is conceivable however, that pilus

detachment may occur at other stages in the bacterial life cycle (14).

The experiments of Table 4 show that F pilus loss can also be observed in cell cultures which are not infected with phage. Cells which were chilled for 15 min at 0 C to inhibit new pilus formation (3) and then transferred to 37 C exhibited a loss and decrease in the length of their F pili at sensibly the same rate as the infected cultures. These results suggest that normally an equilibrium exists between pilus loss and regrowth. By chilling, the process is unbalanced and net pilus loss, i.e., resorption, can be observed. Thus, pilus resorption may be a normal cellular phenomenon which occurs both in the presence and absence of phage. However, the experiments are not definitive, and it is also possible that the cold treatment functions to trigger the withdrawal of the pilus into the cell.

The resorption of the F pilus by the cell during infection with a filamentous bacteriophage suggests a similar function for these structures during bacterial conjugation. A number of experiments has been reported, however, which are not consistent with this interpretation. Brinton has studied the formation of mating pairs under the light microscope and suggested that under some conditions direct wall-to-wall cell contact is not required for the transfer of genetic information during mating (4). These results are of a somewhat qualitative nature but have been confirmed in a series of more elaborate experiments by Ou and Anderson (16). In these experiments, a micromanipulator was used to isolate individual cells from mating pairs which were under continuous observation in the light microscope. The results show that exchange of genetic information can occur between pairs of cells which are never visibly in close contact. The experiments can best be explained if DNA is transferred via the donor F pilus during bacterial conjugation. It seems likely then that DNA transfer in bacterial conjugation and in phage infection occurs by different mechanisms, although both involve the same host structure. Conceivably, the F pilus functions in the transport of DNA only in one direction, and phage infection must consequently occur by another route.

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