Effects of Growth Inhibitors and Ultraviolet Irradiation on F Pili

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The effects of chloramphenicol, nalidixic acid, mitomycin C, NaCN, and ultraviolet irradiation at 253.7 nm on F pilus production by Escherichia coli cells was studied by electron microscopy. The results show that cells contain pools of pilus protein, and that assembly does not require synthesis of protein or deoxyribonucleic acid (DNA). NaCN (2 × 10⁻⁴ M) prevents the reappearance of pilus and causes existing pilus to disappear quickly from the cell surface. This suggests that energy is used in the assembly of pilus and to retain pilus on the cell. Cells irradiated with high doses (10⁶ ergs/mm²) of 253.7 nm light produce fewer pilus, and these are shorter than normal. Dose-response curves for numbers of pilus per cell and length of pilus resemble single hit kinetics, showing 37% survival at 10⁶ ergs/mm² and 2 × 10⁶ ergs/mm², respectively. This suggests that DNA is at the site where pilus are produced, and that it may be involved in the assembly of pilus.

F pilus are filaments up to 2 μm long and are produced by bacteria containing the sex factor F (6). At least eleven genes on the F factor may be involved in genetic transfer, and several of these are undoubtedly involved with the production of pilus (1, 20, 27). F pilus seem to consist of one protein (F pilin) that contains two moles of phosphate and one mole of glucose per molecule (5). Negatively stained pilus appear to have an axial hole or a groove about 2.5 nm in diameter (4, 13, 25); but aside from this and the fact that they are flexible filaments, their structure is not clear. The evidence that F pilus are required for bacterial conjugation and male phage infection and serve as channels for nucleic acid in both processes has been extensively reviewed by others (5, 7, 16, 17, 24).

We are interested in the physiology of F pilus and have tested inhibitors of protein and deoxyribonucleic acid (DNA) synthesis, energy poisons, and ultraviolet irradiation for their effects on pilus. The results reported here show that elongation and retention of pilus require energy but not the synthesis of protein and DNA. Elongation is inhibited by high doses of irradiation at 253.7 nm, suggesting that DNA may be at the site where F pilus are assembled.

MATERIALS AND METHODS

Bacteria and bacteriophage. F pilus were studied in a strain of Escherichia coli K-12 (PT3) which is Fₐlac⁺/lac⁻, thy⁻, thr⁻, leu⁻, met⁻ and produces F pilus, type I pilus, and flagella. PT3 was constructed by introducing Fₐlac⁺ from E. coli C900 into an autotrophic F⁻ strain obtained from D. Freifelder. In some experiments, an Hfr strain of E. coli K-12 (W1895) and an Fₐlac⁺/lac⁻ strain of E. coli B/r (HB11) were used to study pilus. These strains are described in detail elsewhere (19). The ribonucleic acid (RNA) bacteriophage, R17, was used to label F pilus for electron microscopy.

Growth conditions and media. Bacteria were grown aerobically, in either an enriched medium Z (19) or a glucose-salts medium supplemented with Casamino Acids and thymine (GCT medium). GCT medium contains in grams per liter: MgSO₄.7H₂O, 0.025; (NH₄)₂SO₄, 1.0; K₂HPO₄, 7.0; KH₂PO₄, 3.0; sodium citrate·2H₂O, 0.5; glucose, 2.0; Casamino Acids (Difco), 0.1; thymine, 0.1. Cell concentrations were determined from turbidity measurements made with a colorimeter (19). Bacteriophage R17 was grown on W1895, as previously described (19).

Kinetics of reappearance of F pilus. Cultures 50 to 100 ml in volume and containing 5 × 10⁵ cells/ml in the exponential phase of growth were rapidly chilled to 0 C. F pilus were removed from these cells by blending 50-ml samples at 0 C in a homogenizer, as previously described (19). These deiliated cells were then separated from the culture media containing free pili by centrifuging 20-ml samples at 5,000 × g for 15 min at 0 C. Cell pellets were quickly suspended in 20 ml of homologous growth media at 37 C containing the inhibitor to be tested and incubated aerobically at 37 C. At various times, 1-ml samples were removed and quickly chilled to 0 C to halt the growth of F pilus. Blended cells suspended in
growth media at 0 C only have about 0.01 F pilus per cell, as compared to 0.6 pilus per cell for cells that were allowed to grow new pilus for 20 min at 37 C (see Fig. 1). Therefore, it was assumed that our method of blending removed all the F pilus, and that the few remaining pilus were really free pilus stuck to cells.

**Measuring F pilus.** F pilus were assayed by electron microscopy in all experiments. The effects of inhibitors on either the average number of pilus per cell or the average length of attached pilus, or both, were determined from electron micrographs by established procedures (18, 19). To obtain the average number of pilus per cell, at least 100 cells in micrographs were selected at random and measured with a 10× calibrated magnifier.

**Measuring type I pilus.** Type I pilus were assayed on shadowed grids. The length of type I pilus was determined by measuring the distance from the cell surface to the dense portion of the pilus fringe (18). Each value is the average of at least 10 measurements. The fraction of cells with type I pilus was determined by selecting 100 cells at random and counting for the presence or absence of type I pilus.

**Preparation of samples for electron microscopy.** Phase R17 (10 plaque-forming units/ml) and formaldehyde (final concentration 3.7%) were added to samples of cultures at 0 C and then incubated for at least 10 min to permit phage adsorption. A drop was then placed on a copper specimen grid coated with Formvar, and the cells were allowed to settle on the grid for 30 min. The drop was removed with a pipette, and the grid was dried at 60 C for 10 min. Grids were washed in distilled water for 10 sec if the sample was in Z medium, and 2 min if the sample was in GCT medium. Excess water was removed by touching the grid to filter paper, and the grid was placed in a drop of 1% uranyl acetate for either 10 sec or 2 min, depending on whether the original growth medium was Z or GCT. After staining, the grids were dipped into distilled water for a few seconds and dried at room temperature. Grids were examined in an electron microscope (Phillips EM300) at a magnification of about 2,800. Areas on each grid containing an even distribution of cells were photographed, and the micrographs were used to assay F pilus.

In some experiments, grids were shadowed instead of stained. Preparation was the same as above, except staining and succeeding steps were eliminated. Grids were shadowed with platinum in a Kinney metal evaporator at an angle of 14°.

**Ultraviolet irradiation.** Cells were grown and prepared as usual, except samples were irradiated at 0 C just after blending. To irradiate cells, 50 ml of culture was stirred continuously in a 1,000-ml beaker which was kept cold with ice. The source of irradiation was a mercury G15T8 germicidal lamp (General Electric) which emits its energy in discrete bands, with 99.9% of its relative energy at 253.7 nm. Dosage was determined from a calibration curve provided to us by R. M. Klein.

**RESULTS**

**Effect of protein synthesis on the reappearance of F pilus.** Depililated PT3 cells rapidly produce new F pilus when they are resuspended in fresh growth medium at 37 C (Fig. 1). From 0 to 5 min, the number of pilus, expressed as the average number of pilus per cell, rapidly increased from 0 to 0.4 pilus per cell and then increased gradually to 0.6 pilus per cell at 20 min. These kinetics represent our control curve and are typical for F pilus on E. coli cells (4, 18, 19).

Two pellets of depililated PT3 cells were resuspended in media containing chloramphenicol (CM) to study the return of F pilus when protein synthesis was inhibited, and one of these cultures was blended a second time at 10 min. The data (Fig. 1) show that when protein synthesis is inhibited, the number of pilus per cell continues normally for about 10 min and then begins to decrease. Cells in the culture that was blended a second time were still producing pilus, but at a reduced level, similar to that found for cells in CM that were left undisturbed. The average length of pilus made during growth with CM was similar to lengths of control pilus, implying that elongation does not stop when protein synthesis is blocked. The effect of blocking protein synthesis seems to be primarily on the initiation of pilus outgrowth. Similar patterns of pilus production (data not shown) were observed when tetracycline (25 μg/ml) and streptomycin (25 μg/ml) were used.

![Fig. 1](http://jb.asm.org/) Effect of chloramphenicol (CM) on the reappearance of F pilus. Cells were grown in Z medium at 37 C and blended at 0 C. Pellets were suspended in warm media with CM (x) and in media with 500 μg of CM per ml (● and ○). One culture (●) was blended again from 10 to 12 min at 37 C.
to inhibit protein synthesis in PT3 cells, as well as when other cells (W1885, HB11) were used. These results show that cells have pools of F pilin and any other protein required to make a pilus, and that these reserves are large enough under these conditions to sustain a normal rate of pili production for about 10 min. The decrease observed after 10 min seems to reflect a shift of equilibrium, where pili are disappearing from the cell surface faster than they are being produced, possibly because sites for the production of pili become inactive due to depletion of some critical protein.

Inhibition of DNA synthesis and the reappearance of F pili. It seemed pertinent to determine whether or not synthesis of DNA was necessary for pili production, since pili may conduct bacterial DNA during conjugation (4, 6), and conceivably DNA might be associated with pili even in the absence of conjugation. DNA synthesis was inhibited in depiliated PT3 cells with mitomycin C (MitC), nalidixic acid, and by starvation for thymine. None of these treatments had any demonstrable effect on the reappearance of pili (Fig. 2, 3). Normal amounts of pili were produced after 10 min with the inhibitors and after the cells had been starved for thymine for 10 min. The number of F pili per cell is reduced when cells are grown in GCT medium instead of Z medium (compare Fig. 2 and 3). This is consistent with another report that cells produce more pilus when they are grown in enriched medium versus a defined glucose-salts medium (8). Furthermore, the pili produced by these

![Fig. 2. Effect of nalidixic acid and mitomycin C on the reappearance of F pili. Cells were grown in Z medium at 37 C and blended at 0 C, and pellets were suspended in warm media at 37 C (x) or in warm media containing 100 μg of nalidixic acid per ml (O, •) or 50 μg of mitomycin C per ml (Δ, △). Two cultures (• and Δ) were blended again for 10 to 12 min at 37 C.](image)

![Fig. 3. Effect of thymine starvation on the reappearance of F pili. Cells were grown in GCT medium at 37 C and blended at 0 C, and pellets were suspended in GCT medium (x) and in GCT medium minus thymine (O and •). One culture (•) was blended again from 10 to 12 min at 37 C.](image)
phage to pili at 37 °C, thereby making the unlabeled pili difficult to see in our pictures, we examined platinum-shadowed cell preparations that did not contain phage. Many F pili could be seen on control cells, and very few were visible on cells treated with NaCN, dinitrophenol, or NaN₃, which substantiates the claim that no pili are present on poisoned cells.

It is difficult to determine directly whether or not CN prevents R17 attachment to pili at 37 °C, because cells lose their pili under these conditions. We studied R17 attachment to free pili (in supernatant fluids of cell cultures) in the presence of 10⁻² M CN at 37 °C and observed normal attachment in electron micrographs. This, coupled with the fact that R17 phage adsorb to pili at 0 °C (4), makes it unlikely that energy poisons have any effect on attachment.

**Effect of ultraviolet irradiation on the reappearance of pili.** PT3 cells exposed to a high dose (10⁴ ergs/mm²) of ultraviolet irradiation at 253.7 nm produced fewer and shorter pili (Fig. 5). The differences between control cells and irradiated cells in average number of pili per cell and average length of pili after 10 min were about 45 and 25%, respectively. Irradiated cells that were blended a second and third time at 10 and 20 min produced new batches of pili, reduced in number and length from the first batch that was produced between 0 and 10 min. The reduction in number of F pili per cell and length of F pili is caused by irradiation and not by blending the culture three times. Control experiments (data not shown) show that repeated blending of cells grown in Z medium has no effect on the ability of cells to produce normal amounts of F pili. It appears, therefore, that irradiation influences all the pili produced, including those made 30 min after irradiation. A dose-response curve for average length of F pili, average number of F pili per cell, and average length of type I pili is shown on Fig. 6. For F pili, the data resemble single hit kinetics; the 1/e dose for average length and number of pili per cell being 4 × 10⁴ ergs/mm² and 1.5 × 10⁴ ergs/mm², respectively. The resistant fraction depicted in both curves for F pili most likely represents some free pili which remain attached to the cells and possibly bits of debris which adhere to the cells and are scored as short pili in electron micrographs.

As there are many ways in which high doses of ultraviolet irradiation damage cells, it seemed probable that the inhibition was not unique to F pili and that irradiation would inhibit elongation of other surface structures. We examined type I pili on PT3 cells and found that elongation of type I pili is resistant to doses of ultraviolet irradiation that affect F pili.

**DISCUSSION**

Although we cannot construct a detailed model for the growth of pili from information now available, some general characteristics can be surmised from our results and the findings of others.
There is evidence that mixing of F pilin within the pool occurs. Lawn et al. (14) showed cells infected with two F type sex factors, each coding for a serologically distinct pilus, produced hybrid pili that bound both types of F pili antibody. These pili also have a buoyant density intermediate between the two original ones, suggesting that they are hybrid (2).

We presented evidence that energy poisons prevented reappearance of pili and made them disappear from the surface. A similar loss of pili occurs if energy production is blocked with arsenate (R. J. O'Callaghan, personal communication). Energy poisons probably prevent pili from reappearing by blocking an energy-dependent step in assembly. Possibly this step is the phosphorylation of F pilin, which might occur as the subunits are assembled (5). It is not clear how energy poisons cause existing pili to be lost. It is unlikely that termination of assembly alone is responsible, because pili remain on cells if assembly is stopped by cold temperature and formaldehyde. Perhaps other reactions continue in the absence of phosphorylation at 37°C, but not at 0°C or when formaldehyde is present, and these reactions cause rearrangements in assembly sites and the release of pili.

It is also possible that pili retract (16) and are not released following elongation as suggested by Novotny et al. (18). Pili might move in and out of the cell continuously and not require a stimulus such as phage attachment (16) or mating (7) to trigger retraction. Bradley (3, and personal communication) has suggested that the pili on Pseudomonas aeruginosa might do this. The different lengths of F pili which are always observed (4, 18, 21) would be expected if a state of equilibrium existed between pili coming in and going out. Energy poisons might only stop outgrowth, so pili would still retract and quickly disappear. We do not have sufficient data to decide between release or retraction. Our results, however, provide an explanation of how energy poisons inhibit RNA phage adsorption to cells, seemingly to pili. Wendt and Mobach (26) showed that energy poisons inhibited the binding of radioactive RNA phage (f2) to attached pili but not to free pili, and suggested that pili were changed to a new state. We agree, and add that this state represents the disappearance of pili from the cell surface.

It seems pertinent to consider the energy requirement of mating-pair formation in terms of an effect on F pili. Fisher (10) showed that genetic transfer required energy, and suggested that donor cells needed energy to form mating pairs. If pili are required for mating-pair for-
Inhibition, energy would be needed at least to make and retain pili. Curtiss and Stallions (9) found that 2 x 10⁻⁴ M KCN did not inhibit mating-pair formation, and concluded that specific pair formation does not require energy. We find that 2 x 10⁻⁴ M NaCN is relatively ineffective in making pili disappear: about 60% of the pili still remain on cells after 3-min incubation with 2.5 x 10⁻⁴ M NaCN. It will be interesting to see if higher concentrations of CN (1 x 10⁻³ M), which make pili disappear, prevent pair formation.

High doses of ultraviolet irradiation at 253.7 nm inhibit the reappearance of pili, and those pili which return are shorter than normal. It is not clear what the sensitive target is, so we can only speculate on the cause of inhibition. The inhibition is not general, affecting the growth of all surface structures, because type I pili are resistant. Also, it is unlikely that photochemical damage to protein, like the induction of protein-protein cross-links, is responsible for the inhibition, because type I pili, like F pili, consist of a single protein and are assembled from pools of subunits (4). Therefore, something is different about F pili which makes them sensitive to irradiation. Most likely F pili are associated with DNA, and damaged DNA inhibits elongation of F pili. Our experiments with inhibitors of DNA synthesis show that DNA synthesis is not required for the reappearance of pili, but DNA could be at the site where pili are assembled. Doses of ultraviolet irradiation, in the range used to inhibit F pili, are known to cause pronounced cross-linking between complementary strands of DNA (15) and to induce DNA to bind covalently to protein (23). Perhaps damage of this type inhibits elongation of F pili. Formation of dimers between adjacent thymines is probably not important here, since low doses of irradiation which induce them have little effect on pili.

We can only speculate on how DNA might be associated with pili. It is possible that certain genes on the sex factor are associated with the site where a pilus is assembled. Irradiation might induce pilus proteins to bind to DNA, making them unavailable for assembly. Assembly might be blocked completely at some sites so fewer pili are produced following irradiation. At other sites, assembly might occur, but because there are fewer subunits, short pili are produced. Another possibility is that pilus proteins assemble around a single-strand section of sex factor DNA, whose length determines the length of the pilus. DNA-DNA cross-links between complementary strands would prevent strand separation and would reduce the length of single-stranded DNA used in assembly so short pili would be produced.

Two other findings which will be reported elsewhere are consistent with DNA being associated with pili. PT3 cells were grown for several generations in Z medium with 100 μg of 5 bromouracil (5BU) per ml, which allows for maximal incorporation of 5BU into DNA (28). F pili produced by these cells have an average length which is twice that of normal pili and are more sensitive to ultraviolet irradiation with regard to length (P. Taylor and C. Novotny, in preparation). The increased sensitivities of pili length to irradiation is consistent with DNA being the target, since 5BU incorporation into DNA makes DNA more sensitive to photochemical change (12). The twofold increase in average length of pili which occurs when 5BU incorporation into DNA is maximal suggests that DNA is associated with pili and can influence the average length of F pili.

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LITERATURE CITED


