*A NEW TYPE OF BACTERIAL PILUS GENETICALLY CONTROLLED BY THE FERTILITY FACTOR OF E. COLI K 12 AND ITS ROLE IN CHROMOSOME TRANSFER**

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Pili comprise several types of morphologically similar thin appendages growing out from the surface of gram-negative bacteria.[1-3] Type I pili are composed of protein subunits of molecular weight 17,000 polymerized into rigid right-handed helices of diameter 70 Å and pitch 24 Å, having an axial hole 20–25 Å in diameter.[4] Other types of pili with different external diameters exist[2] but their composition and fine structure is unknown.

It had been considered previously that pili might be involved in the fertility of male bacteria.[3] This speculation was based on the frequent occurrence of pili on male *E. coli* K 12 strains and on the plausibility of chromosomal transfer being mediated by a rod-like structure which could actively traverse the cell membrane and wall. However, no correlation of piliation with maleness was found. Many female strains were richly piliated and some cultures of male strains contained only a few sparsely piliated cells. The hypothesis could not be disproved, however, since none of the many male strains subsequently examined were ever completely non-piliated. Although the majority of cells in a culture may have had no pili, at least a few cells could always be found with a few attached pili.

A new method of approach to this problem has been provided by the recent electron microscopic observations of Crawford and Gesteland[5] who noted that a male-specific bacteriophage, R-17, adsorbed to pili of an Hfr and an F^+ strain of *E. coli* but not to pili of an F^- strain.

We have investigated the adsorption of another male-specific bacteriophage, M 12, isolated by P. H. Hofschneider.[6] Our studies revealed that M 12 adsorbs to some of the pili present on male bacteria and that phage adsorption can be used to distinguish them in electron micrographs from other types of pili occurring on the same cell. It is possible to demonstrate that these "F pili" are genetically controlled by the fertility factor of *E. coli* K 12.

Materials and Methods.—Phage: M 12 phage, a small (about 270 Å diameter), spherical, RNA-containing phage infecting Hfr and F^+ strains but not F^- strains, was obtained from Dr. P. H. Hofschneider.

Electron microscopy: Bacteria were grown in tryptone yeast extract calcium broth (10 gm tryptone, 5 gm yeast extract, 5 gm NaCl, 0.75 gm $CaCl_2 \cdot 2H_2O$ per liter of water, the $CaCl_2$ sterilized separately). Overnight, unshaken, unaerated cultures were diluted 1:10 into fresh medium and grown for 3–4 hr under the same conditions until the bacterial density was about $5 \cdot 10^8$ cells per ml. M 12 phage was added at multiplicities from 5 to 100 and the mixture incubated at 37°C. After 10 min, the mixture was rapidly chilled in an ice bath and prepared for the electron microscope by the collodion agar filtration method of Kellenberger.[7]

Tests for phage susceptibility: (A) *Plaque formation:* Standard phage plaque methods using tryptone yeast extract calcium soft agar (0.7%) and bottom agar

(1.5%) were employed. The plaques varied from turbid to clear, depending on the strain of bacteria and time of incubation.

(B) *Titer increase:* Some strains of bacteria which formed no plaques with M 12 were tested for their ability to replicate and release M 12. Exponentially growing cultures were infected at a multiplicity of 0.1 and incubated with aeration at 37°C overnight. Chloroform and lysozyme were added and the cultures were reincubated for 30 min. Each culture was centrifuged to remove bacteria and the supernatants were assayed for M 12 plaques using *E. coli* W1895 Hfr as indicator bacteria.

Results.—Piliation of Hfr, F+, and F− strains: An electron microscopic examination of 14 Hfr, 2 F+, and 2 F− strains of *E. coli* K 12 (Table 1, Fig. 1) showed all the

TABLE 1

SURVEY OF STOCK STRAINS FOR F PILI, TYPE I PILI, FERTILITY, AND M 12 PHAGE SUSCEPTIBILITY

E. coli K 12 strain	Source	F status as given	Fertility[a]	M 12 phage susceptibility[b]	F pili[c]	Type I pili[d]
W1895	L. S. Baron	Hfr	+	+	+	+
W1485	"	Hfr	*	+	+	+
199 Hfr HCB	H. C. Boyer	Hfr	*	+	+	+
AB 693	E. Englesberg	Hfr	+	+[e]	+	+
AB 347	"	Hfr	*	+	+	+
P4x6	"	Hfr	+	−	+	+
P 10	"	Hfr	+	+	+	+
AB 750	"	Hfr	+	+	+	+
AB 353	"	Hfr	*	+[e]	+	+
AB 663	"	Hfr	+	+	+	+
AB 373	"	Hfr	*	+	+	+
AB 378	"	Hfr	*	+	+	+
AB 379	"	Hfr	*	+	+	+
AB 311	"	Hfr	*	+	+	+
58-161	L. S. Baron	F+	+	+	+	+
200 U	"	F+	*	*	+	+
P 678	W. Maas	F−	−	−	−	+
200 U	L. S. Baron	F−	*	*	−	+

* Not tested in our laboratory.
[a] Ability to donate lac+ to *E. coli* Bam P−lac−ara− (derived from *E. coli* Bam P+lac+ara+).[11]
[b] Plaque formation with M12 phage.
[c] Pili adsorbing M 12 phage (electron microscopy).
[d] Pili not adsorbing M 12 phage (electron microscopy).
[e] 500–1,000-fold titer increase but no plaque formation. Virus is replicated and presumably released without significant lysis.

Hfr and F+ strains to have pili capable of specifically adsorbing M 12 phage. It is proposed to call these structures "F pili" because of their morphological similarity to Type I pili and their genetic determination by the F factor. The F− strains produced no detectable F pili. All of the strains, including the F− strains, also produced Type I pili which could not be distinguished from F pili in the absence of added M 12 phage. However, phage adsorption clearly discriminates between the two types since M 12 at high multiplicities will entirely cover the F pili without noticeably interacting with Type I pili on the same bacterium.

Although there may be as many as three or four hundred Type I pili on a single cell, the number of F pili per cell varies from none to three or four, the most usual numbers being one or two. This distribution may be influenced by growth and preparation of the bacteria for microscopy since unattached F pili are found in most preparations. The length of F pili varies from a few tenths of a micron to ten microns or more. There seems to be no preferred point of attachment, both sides

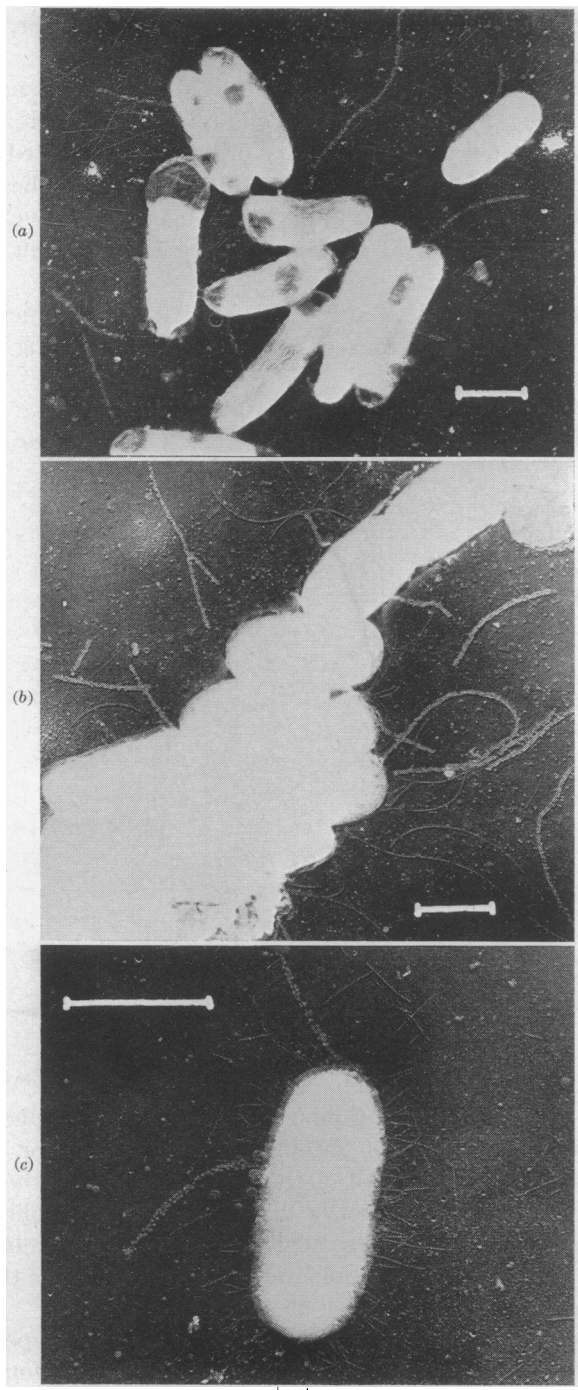

FIG. 1.—White lines are 1 μ. (*a*) *E. coli* K 12, W1895, Hfr (Cavalli) + M 12 phage. F pili are distinguishable from Type I pili by the presence of adsorbed M 12 phage. (*b*) *E. coli* K 12, 58-161, AO-1, Mg13, F^+ + M 12 phage. This strain, originally F^+ and having both F pili and Type I pili, had lost its F pili by acridine orange elimination of the fertility factor. Upon reintroduction of the fertility factor by infection, the F pili reappeared. (*c*) *Proteus mirabilis* F-lac^+ + M 12 phage. This strain, originally F^- and lacking F pili but having Type I pili, acquired F pili and fertility upon introduction of the *E. coli* fertility factor.

and ends of the cell serving as apparent points of F pili origin. The F^+ strains and the Hfr strains had approximately equal numbers of F pili. The distribution of F pili on an Hfr and an F^+ strain determined by a microscopic count of free and attached F pili without regard for length is shown in Table 2.

TABLE 2

DISTRIBUTION OF F PILI IN MICROGRAPHS OF *E. coli* K 12 W1895 Hfr AND *E. coli* K 12 58-161 AO-1 #12 F^+

				Hfr			
No. F pili on cell	0	1	2	3	4	5	More than 5
No. bacteria	95	52	16	4	0	0	0
Fraction of total	0.57	0.31	0.096	0.024			
No. of unattached F pili = 28 per 167 bacteria							
				F^+			
No. F pili on cell	0	1	2	3	4	5	More than 5
No. bacteria	61	42	14	2	1	2	0
Fraction of total	0.50	0.34	0.11	0.016	0.008	0.016	
No. of unattached F pili = 64 per 122 bacteria							

Elimination of F pili by acridine orange: The microscopic survey strongly suggested an association of F pili with the presence of the fertility factor. To test this hypothesis, *E. coli* K 12 58-161 F^+ was converted to F^- by growth in the presence of acridine orange (AO).[8] Five ml of Hirota broth containing 20 μg per ml of AO was inoculated with about 10^4 cells of 58-161 F^+. As a control, a similar inoculation was made into Hirota broth without AO. After overnight incubation at 37° without shaking, aliquots were plated on nutrient agar. Following incubation of the plates, 51 colonies from the AO culture and 30 colonies from the control culture were picked at random and inoculated into individual tubes of nutrient broth. All 81 of these cultures were tested for fertility the following day by mating with *E. coli* Bam $P^-lac^-ara^-$ recipients on minimal lactose agar, selecting for lac^+ recombinants. About 5×10^7 58-161 donor cells were cross-streaked with about 5×10^7 Bam $P^-lac^-ara^-$ recipient cells and scored for recombinants after 48 hr incubation. A 58-161 donor clone was considered F^- if no lac^+ recombinants were evident at this time. Fifty of the 51 AO clones were F^- while 3 of the 30 control clones were F^- by this test. All of the 12 F^- AO clones examined had no F pili and formed no plaques with M 12, while all of the 7 F^+ control clones examined had the usual distribution of F pili and formed plaques with M 12. Both the AO clones and the control clones had similar distributions of Type I pili. Thus, elimination of the genetic factor controlling fertility also eliminated F pili and male phage susceptibility but did not affect Type I piliation.

Addition of F pili by infection with F factor: To further test the hypothesis that F piliation is genetically controlled by the F episome, the F factor was introduced by infection into an F^- recipient during mixed cultivation in nutrient broth. Cultures of 58-161 F^+ *Str*^{s} and P678 F^- *Str*^{r} were grown separately overnight in nutrient broth. The F^+ cells ($2–5 \times 10^5$) and the F^- cells (10^4) were inoculated into 5 ml of nutrient broth and grown together overnight at 37°C without shaking. Separate control tubes inoculated with 10^4 F^- cells or $2–5 \times 10^5$ F^+ cells were also grown overnight. Aliquots from the three tubes were then streaked on nutrient agar plates containing 500 μg/ml of streptomycin. The control culture of 58-161 F^+ *Str*^{s} produced no colonies on the streptomycin agar. Sixty P678 recipient colonies

from the mixed culture and 35 colonies from the pure P678 F$^-$ *Str*^{r} culture were picked and inoculated into 2.5-ml volumes of nutrient broth and incubated without shaking overnight. These 95 cultures were tested for fertility by mating with Bam P$^-$lac$^-$ara$^-$ and selecting for lac$^+$ recombinants. Forty-eight of the 60 P678 clones isolated from the mixed culture had acquired fertility while none of the 35 control P678 clones were fertile. All 10 of the P678 F$^+$ *Str*^{r} clones examined had the usual distribution of F pili and formed plaques with M 12, while all 5 of the P678 F$^-$ *Str*^{r} control clones examined had no F pili and formed no plaques with M 12. Both F$^+$ and F$^-$ clones had similar distributions of Type I pili. Thus, acquisition of the fertility factor resulted in the appearance of F pili and male phage susceptibility while the status of Type I piliation was again unaffected.

A similar experiment was performed using a different recipient, *E. coli* K 12 58-161 AO-1 F$^-$ *Str*^{r}, and the same F$^+$ donor strain. Sixteen of the 20 AO-1 clones from an overnight mixed culture plated on streptomycin agar were F$^+$. Nine of the F$^+$ clones were chosen for examination and all were found to have F pili. The six control F$^-$ clones that were examined had no F pili. Type I piliation was unaffected.

A convincing verification of F factor control of F piliation was obtained by the examination of strains in genera and species other than *E. coli* K 12 into which *E. coli* K 12 F factors had been introduced.[9] Six strains lacking F pili have been found to acquire them by the introduction of *E. coli* K 12 F factor (Table 3).

TABLE 3

THE PRESENCE OF F PILI IN OTHER GENERA, SPECIES, AND STRAINS CONTAINING AN EXPERIMENTALLY ACQUIRED *E. coli* K 12 F FACTOR

Strain and characteristics	Source	F status as given	F piliation
Salmonella typhimurium LT-2 SR 279	L. S. Baron	F$^+$	+
Salmonella typhimurium LT-2 (parent)		F$^-$	−
Salmonella typhosa 643 F-lac	S. Falkow	F-lac$^+$	+
Salmonella typhosa 643 (parent)		F$^-$	−
Shigella flexneri 69	H. Schneider	Hfr	+
Shigella flexneri 2457T (parent)		F$^-$	−
E. coli B/r HB11 F-lac	H. C. Boyer	F-lac$^+$	+
E. coli B/r (parent)		F$^-$	−
Proteus mirabilis F-lac	L. S. Baron	F-lac$^+$	+
Proteus mirabilis PM-1 (parent)		F$^-$	−
Shigella flexneri Ron 1 Hfr	H. Schneider	Hfr	+
Shigella flexneri 5 F$^-$ (parent)		F$^-$	−

The F$^-$ parental strains into which the F factors were introduced were also examined as controls.

Discussion.—The microscopic observations of Crawford and Gesteland demonstrated that the male-specific phage, R-17, could adsorb to pili produced by an Hfr male and an F$^+$ male but not to those of a female strain of *E. coli* K 12. This led them to suggest that attachment to pili might represent the initial step in R-17 replication. They also observed that in conjugating mixtures of *Hfr* and F$^-$ bacteria, the male phage attached to the pili of only one of the two mating partners. Although not explicitly stated by these authors, these observations implied the existence of two different kinds of pili.

Using another male-specific phage (M 12) and a large number of Hfr, F$^+$, and F$^-$ strains, our experiments have demonstrated the existence of two types of pili under independent genetic control. That the synthesis of pili capable of adsorbing M 12

is determined by the *E. coli* F factor is shown by the correlation of F piliation with the presence of the F factor in derivatives of *E. coli* K 12, the disappearance of F pili when the F factor is removed, and the appearance of F pili when the F factor is added. On the other hand, the genetic control of Type I piliation is chromosomal rather than episomal[10, 11] and by the method of interrupted mating has been located 2 min from the threonine marker on the side away from leucine. A given cell may produce either Type I pili, F pili, both, or neither. This is the first demonstration that the same cell can produce more than one kind of pili.

Earlier observations that piliation was not correlated with fertility[3] can now be understood. F pili are much less numerous than Type I pili and cannot be distinguished from them in electron micrographs in the absence of male phage adsorption. The pili occurring on F^- strains are Type I pili only, and are not correlated with fertility.

The results presented here strongly suggest that F pili are identical to the f^+ surface antigen of Ørskov and Ørskov.[12, 13] In general, the f^+ antigen occurs only on Hfr and F^+ strains and not on F^- strains. The fact that the average number of F pili per cell is about one (due presumably to the small original number, subsequent breakage during culture and spontaneous loss of F factor) could explain why the f^+ agglutination reaction is weak. Ørskov and Ørskov had difficulty in detecting the f^+ antigen of *E. coli* K 12 perhaps due to masking of the f^+ agglutination reaction by Type I pili which occur abundantly in this strain. Our finding that Hfr and F^+ strains have similar numbers of F pili correlates well with the approximately equal amounts of f^+ antigen found on Hfr and F^+ strains by Ørskov and Ørskov.

An important question raised by these studies is whether or not F pili are directly concerned with fertility, that is, the formation of specific mating pairs and the ordered intercellular transfer of chromosomal or episomal DNA. The apparent pleiotropic relationship between fertility and F piliation is consistent with the hypothesis that F pili are necessary for fertility but could also mean that the F pili genes are simply linked to the *E. coli* K 12 F factor and have no physiological function in chromosome transfer.

Several functions controlled by the F factor can be separated by mutation. Nishimura and Hirota[14] have isolated an F′ strain with a mutant F factor which is fertile but resistant to male-specific phages. As shown by the methods used in this study, strain P4x6 was fertile and produced F pili but was insensitive to M 12 phage. Lederberg and Lederberg[15] have isolated F refractory mutants which were sterile but sensitive to male-specific phage f_1. Sterile mutants having f^+ antigen have been isolated by Ørskov and Ørskov.[12, 13] However, the existence of these mutants does not eliminate a direct role of F pili in phage infection or the mating process, since these functions could be lost by mutation without affecting F piliation if the presence of F pili is necessary but not sufficient for these functions. A direct role of F pili could be disproved if mutants could be found which retained fertility but lost F pili. To our knowledge, no fertile mutants have been isolated which have been demonstrated to lack F pili or the f^+ antigen.

Preliminary experiments have provided direct evidence that F pili are necessary for chromosome transfer.[16] Phenotypic removal of F pili from an Hfr donor by mechanical agitation prior to a brief period of mating pair formation reduced the number of subsequently formed recombinants to less than 0.1 per cent of the F

piliated control. Under optimum physiological conditions, F pili were rapidly regenerated, and the ability to transfer chromsome returned proportionately. Transfer ability did not return if F pili were not regenerated. Both transfer ability and F piliation were reduced when the male culture was grown in a poor medium or with vigorous aeration. In addition, the processes of chromosome transfer and F pili regeneration are separable, since F-piliated male cultures in the stationary growth phase were able to transfer chromosome efficiently but were not able to regenerate new F pili after the original F pili were removed. It is not yet known whether F pili are necessary for specific pair formation, effective pair formation, or for both.

The extent of structural similarity between Type I pili and F pili is only partially known, but their identical appearance in the electron microscope with respect to outer diameter and axial hole diameter makes further structural similarities likely. If one assumes F pili to be similar to Type I pili, that is, protein rods with an axial hole 20–25 Å in diameter, actively growing from inside the cell to the outside, some plausible models for intercellular DNA transfer can be imagined. The chromosome might fit into the axial hole of the F pilus and could be carried from male to female (*a*) by remaining fixed inside the F pilus and moving from male to female by outgrowth of the entire F pilus-chromosome complex, or (*b*) by moving actively through the axial hole of the F pilus. Biological analogies exist for both models. Hofschneider[17] has found that a rod-shaped DNA bacteriophage, M 13, 70 Å in diameter, is released from infected cells by growing out through the membrane-cell wall barrier without lysing the cell in a manner analogous to model *a*. The injection of T-even phage DNA through the central core of the phage tail (outer diameter 80 Å, inner diameter 20–25 Å) into the bacterial cytoplasm is analogous to model *b*. Experiments designed to elucidate a direct role of F pili in chromosome transfer are being carried out. Present knowledge indicates model *b* to be more likely since efficient chromosome transfer can take place from male cells unable to produce new F pili.

The relationship of the F pilus to the "conjugation bridge" of Anderson *et al.*[18] remains to be resolved. The F pilus could go through the "conjugation bridge" or could transfer chromosome from cell to cell independently of such a bridge. It is noteworthy that in most published electron micrographs of conjugating bacteria, pili can be seen on the male partner, often going from male to female. It has been observed in this laboratory that "conjugation bridges" appear in electron micrographs between males (Fig. 1*a*) and even between females. In fact, electron micrographs of most full-grown pure cultures of enteric bacteria showed a large proportion of cells adhering to each other, many of them appearing to be connected by "conjugation bridges." These observations raise the possibility that "conjugation bridges" are artifacts of electron microscope preparation and may not be directly related to chromosome transfer.

The finding of Sneath and Lederberg[19] that periodate acts on Hfr and F^+ cells to prevent chromosome and episome transfer led them to postulate the existence of a polysaccharide conjugal substance on the male cell surface. They suggested that the male substance might be identical with the f^+ antigen. Purification of F pili is being carried out in an attempt to determine whether or not they contain polysaccharide or are composed solely of protein, as are Type I pili.

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*ELECTRICAL STIMULATION OF THE INTERNODES OF SINGLE FIBERS OF NERVES WITH INTACT SHEATH**

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In a preceding communication[1] proof was given that in response to stimulation by the outward flow of action currents all points of the internodes of myelinated nerve fibers produce in succession the EMF of the action potential. In this communication proof is given that applied cathodal currents can initiate nerve impulses at all points of the internodes.

Technique.—The experimental observations have been made with the long and slender peroneal branches of the Texan bullfrog (probably a variety of *R. sphenocephala*). In order to detect the response of a single fiber, a method originated by Adrian and Bronk[2] is used. At the bifurcation of the peroneal trunk an opening is made in the external connective tissue sheath and all the nerve fibers are cut, except one large myelinated fiber.