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Some Effects of Temperature on the Growth of F Pili

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The effect of temperature on the production of F pili by an F+ strain of *Escherichia coli* B/r was studied by electron microscopy and by a technique involving serum-blocking power. The latter method is based on the ability of F pili to adsorb F pili antibody which inhibits male-specific phage infection. The total amount of pili in a sample was estimated by serum-blocking power; the length of F pili and number per cell was determined by electron microscopy. Cell extracts prepared by sonic oscillation lacked serum-blocking power, suggesting that F pili are not present in the cytoplasm. The number of F pili per cell varied with the growth temperature, but the average length of F pili remained constant. Maximum number of pili per cell occurs between 37 and 42 C; below 37 C the number decreases, reaching zero at about 25 C. When cells are grown at 37 C, blended, and resuspended in fresh media at 25 C, they make F pili. These pili are probably assembled from a pool of subunits that were synthesized during growth at 37 C. The rates of assembly at 25 and 37 C, as judged by the rate of increase in length of F pili, are similar. When cells were grown at 25 C and shifted up to 37 C, there was a 30-min lag in pili production followed by a period of rapid outgrowth. When cells were shifted down from 37 to 20 C, outgrowth (assembly) of pili ceased, and approximately 50% of the attached pili were released in 2 min. No release was observed when cells were shifted to 0 C. This suggests that pili may be released from the cell by a mechanism that requires metabolic activity, but not the outgrowth of F pili.

F pili are filamentous surface structures produced by F+ and Hfr bacteria (4, 7) and are representative of a class of appendages called "sex pili" (15, 17). The genes for F pili are on the F factor (4) and comprise four contiguous complementation groups (20, 21). One of these complementation groups seems to be a structural gene for F pili, but the functions of the other three genes are unknown. In electron micrographs, pili appear as flexible rods of various lengths that seem to emerge at random sites on cell surfaces (1, 14). Some pili appear to have terminal knobs or bulges, but whether these are parts of pili or merely cellular debris stuck to the pilus tip remains to be determined (14, 29). Negatively stained pili seem to have an axial hole about 2.5 nm in diameter (1, 14, 29), but the exact arrangement of subunits in F pili and their chemical composition are unknown. In addition to being degraded by organic solvents, F pili are digested by several proteolytic enzymes (3, 26, 28). Purified preparations seem to consist mainly of protein plus a small amount of glucose and phosphate (C. Brinton, *personal communication*), and it has been suggested that pili may also contain some lipid material (26).

There is a great deal of evidence that sex pili are required for conjugation and for infection by male phage, but just how the pilus functions is still uncertain (for reviews, see 1, 2, 8, 16, 17, 28). Brinton et al. (4) initially suggested that pili conducted deoxyribonucleic acid (DNA) between mating bacteria, and later it was proposed that pili could conduct male phage nucleic acid (1, 2). Curtiss (8) suggested that the pilus may be a retractable structure that attaches to a female and draws her to the surface of the male cell as it retracts. A similar retraction mechanism was proposed by Marvin and Hahn (16) to explain how filamentous phage can attach to the tip of the pilus (5) and then penetrate the cell intact (25). Some evidence that pili may conduct bacterial DNA was provided by the demonstration by Ou and Anderson (23) that genetic transfer can take place between mating pairs that never make wall-to-wall contact.

Investigators have concentrated mainly on the functional aspects of F pili, and, as a consequence, little is known about the growth of F pili per se. There have been several reports of the

effects of growth conditions on F pili. Cells have been reported to have from one to four pili, depending on the strain and cultural conditions employed (1, 2, 9, 14, 18, 28). Generally, it appears as if maximum piliation is attained at the end of the exponential phase of cell growth, and that cells produce more pili when they are grown in enriched medium versus a defined glucose-salts medium (1, 9, 28).

There is one report of pili production at different growth temperatures. Knolle and Ørskov (13) showed that when cells are grown at 18 C they cannot adsorb phage fr but regain this ability when the temperature is raised to 37 C. This discovery and the observation that the yield of fr plaques decreased at temperatures below 34 C led them to suggest that F pili production was very sensitive to temperature.

We have been studying several aspects of pili outgrowth in our laboratory. In this paper, we present some of our findings concerning the influence of temperature on pili production and a model for the growth of F pili that is consistent with our results. We also report a method for assaying F pili based on serum-blocking power. It has been shown that serum from rabbits immunized with either whole *E. coli* male cells or preparations of sex pili contains antibody that binds along F pili and prevents conjugation and male phage infection (12, 15; C. Brinton et al., Bacteriol. Proc., p. 67, 1969). The assay we developed was based on the ability of a known amount of antibody to inhibit male phage infection before and after adsorption with F pili.

MATERIALS AND METHODS

Bacteria and bacteriophage. Two strains of *E. coli* were used in most experiments: HB11, an F^+ strain of *E. coli* B/r was used to study F pili; W1895, an Hfr strain of *E. coli* K-12 was used to plaque phage. These and other strains of *E. coli* studied are listed in Table 1. We also used two F pili phages: a spherical ribonucleic acid (RNA) phage (R17) and a filamentous DNA phage (M13) originally donated by J. Watson and P. Hofschneider, respectively.

Growth conditions and media. Bacteria were grown aerobically at 37 C in Z medium which contains (in grams per liter): tryptone, 10; yeast extract, 1; NaCl, 8; glucose, 1; and NaOH, 0.3. TB agar medium was used to plaque phage and contains (in grams per liter): Trypticase Soy Broth (BBL), 30; agar (Difco), 15 (hard agar) or 7.5 (soft agar). Cell concentrations were determined from turbidity measurements made with a colorimeter (model 9, Coleman Instruments, Inc., Maywood, Ill.) that had previously been calibrated in terms of viable cells per milliliter.

Preparation of phage stock and phage antiserum. Bacteriophage R17 and M13 were grown on W1895 cells in Z medium and lysates (phage stock) were prepared as previously described (19). Phage antiserum was prepared by injecting 0.5 ml of phage stock containing about 10^{12} plaque-forming units (PFU) per ml, subcutaneously, in the backs of white rabbits. Injections were repeated 1 month later with 0.1 ml of phage stock, and approximately 10 days later the rabbits were bled by cardiac puncture.

Preparation of F pili and F pili antiserum. The F pili antiserum used was a gift from Charles Brinton, Jr., and was prepared as follows. F pili obtained from HB11 were concentrated and purified by the method of C. Brinton, S. Polen, and E. Raizen (*manuscript in preparation*) and injected into rabbits for production of specific antisera. This solution of pili formed a single band in a cesium chloride density gradient, exhibited an ultraviolet (UV) adsorption spectrum to be expected of structures like F pili and was missing several amino acids. Electron microscopy revealed no structures other than long aggregates of F pili rods with some attached and free terminal knobs (C. Brinton, *personal communication*). Male and female *E. coli* cells were agglutinated by 1/10 dilutions of serum; higher dilutions up to 1/50 exhibited some agglutinating activity that was specific for male cells. The nonspecific antibody responsible for agglutinating both male and female cells was removed from serum preparations by adsorption with HB11 F^- cells which is an isogenic strain of HB11 cured of F^+ after treatment with acridine orange (10). Adsorption was done as follows. HB11 F^- cells were grown in Z medium, washed in phosphate buffer (*p*H 7.0), and resuspended in antiserum to a final concentration of 10^{10} cells per ml. The mixture was incubated at 0 C for 60 min, and the cells were removed by centrifugation at 4 C for 10 min at 12,000 × *g*. Fresh cells were added to the supernatant fluid, and the adsorption process was repeated. After this second treatment, the serum was sterilized by filtration. A single batch of serum prepared in this way was used in all of our experiments and will be called F pili antiserum (FAB). A serum preparation, obtained from nonimmunized rabbits, served as control serum (CAB).

A brief description of the inhibition of male phage infection and bacterial conjugation by F pili antibody has appeared (C. Brinton et al., Bacteriol. Proc., p. 67, 1969); a detailed analysis will be presented elsewhere (C. Brinton, E. Raizen, C. Novotny, and S. Polen, *manuscript in preparation*).

Standard test system for phage M13 infection. M13 infection of W1895 cells was carried out in Z medium at 37 C as follows. At zero time, 0.05 ml of W1895 culture growing exponentially in Z medium and containing 5×10^8 cells per ml was added to 0.5 ml of Z medium. Six minutes later, 0.05 ml of an M13 phage lysate that had been diluted in Z medium to a concentration of 10^{10} PFU per ml was added. Infection was allowed to proceed for 6 min, and then the mixture was diluted 1/1,000 into Z medium, M13 antiserum was added, and the mixtures were incubated for 6 min. The M13 antiserum concentration was adjusted so that after 6 min the surviving fraction of input phage was less than 0.01%. The mixture was then diluted 1/10 in Z medium to reduce the M13 antibody concentration, and 0.1-ml samples were assayed for M13-infected cells by the agar overlay technique. The top layer consisted of 3 ml of soft agar with 10^7 W1895 cells per ml added as indicator bacteria. Plates were incubated at 37 C,

and plaques were counted after 6 hr. We considered these PFU to be infective centers having arisen from W1895 cells that were infected with M13, because at least 99% of the PFU detected were resistant to M13 antiserum and sensitive to chloroform. Phage R17 was sometimes added to reaction mixtures instead of M13 as noted in figure legends. Infection conditions for R17 were identical to those for M13 except that R17 antiserum was used.

Inhibition of phage infection by FAB. To measure the inhibition of M13 and R17 phage infection by antibody, different amounts of FAB and CAB were added to infection mixtures containing Z medium and W1895 cells. Six minutes later, either M13 or R17 phage were added, and infection was carried out as described above. The number of infected cells, with each concentration of FAB, is shown in Fig. 1. Similar curves for the inhibition of male phage infection by FAB were observed in other studies (C. Brinton, E. Raizen, C. Novotny, and S. Polen, *manuscript in preparation*).

Serum-blocking power. The serum-blocking power of attached F pili was determined as follows. HB11 cells were grown in Z medium to a cell concentration of 5×10^8 cells per ml, and the culture was chilled to 0 C in an ice bath and centrifuged at $3,000 \times g$ for 15 min. We assume that all the unattached pili known to be present in these cultures (18) remain in the supernatant fluid and that all of the F pili in the pellet are attached to the cells. The pellets which contain attached F pili were resuspended to the desired concentration by adding an appropriate amount of Z medium (0 C) to the centrifuge tubes. Pellets were dispersed by agitation with a Vortex-Genie mixer (Scientific Industry) set at low speed. Samples (0.9 ml) of resuspended cells were placed in glass centrifuge tubes, (18 by 100 mm), and 0.1-ml amounts of FAB that had previously been diluted in Z medium to a desired concentration were added. These mixtures were incubated without agitation for 60 min at 0 C and then centrifuged at 10,000 $\times g$ for 15 min. The supernatant fluids containing unadsorbed FAB were collected, and 0.5-ml samples were used as reaction mixtures for M13 infection. R17 phage was used occasionally as specifically noted in figure legends. In some experiments, the adsorption mixtures were not centrifuged; instead we filtered them through 0.2-μm membrane filters (GA-6 filters, Gelman Instrument Co., Ann Arbor, Mich.) and used the filtrates as reaction mixtures for phage infection. The amount of FAB remaining in either the supernatant fluid or filtrate was determined by calculating the per cent inhibition of phage infection (per cent PFU) and estimating the corresponding antibody titer from the standard inhibition curve shown in Fig. 1.

Estimation of the amount of F pili. The relative amount of F pili in a sample could be estimated from a standard curve describing per cent PFU as a function of cell concentration which should be directly proportional to the amount of attached antigen or F pili (Fig. 2). To obtain the data for this curve, various amounts of HB11 cells were added to adsorption mixtures that contained a constant amount of FAB (0.25 units per ml). These cells were grown in Z medium at 37 C and resuspended in fresh Z as previously described. To calculate the relative amount of F pili in a sample, we determined the per cent PFU for the sample and appropriate control cells after adsorption in mixtures containing 0.25 unit of FAB per ml. The standard curve was then used to estimate the corresponding values for amount of F pili per cell in each sample.

Effect of temperature on F pili outgrowth. In these experiments, HB11 cells were grown in Z medium and chilled to 0 C, and then 50-ml samples were blended for 2 min at 10,000 rev/min in an Omni-Mixer homogenizer (model OM-1150, Ivan Sorvall, Inc., Norwalk, Conn.) with a 200-ml stainless-steel chamber. Blending was carried out at 0 C by immersing the cup in an ice bath. This treatment removes at least 95% of the attached pili (18). The blended cells were then centrifuged in the cold as described above, and the pellets were resuspended in Z medium at the described temperature and incubated by shaking. Samples were removed at various times, chilled to 0 C to prevent further outgrowth of F pili, and assayed for F pili as described. The cell concentration in adsorption mixtures was 4×10^9 cells per ml.

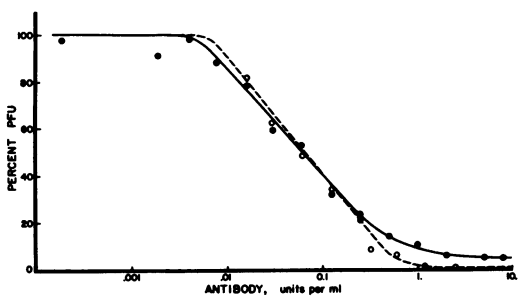

FIG. 1. *Inhibition of M13 and R17 infection by F pili antiserum (FAB) and control serum (CAB). Symbols: O, R17 infection; ●, M13 infection. Per cent plaque-forming units (PFU) equals PFU in presence of FAB divided by PFU in presence of CAB × 100. Undiluted serum equals 100 units of serum per milliliter. Each point represents the average of at least three separate experiments.*

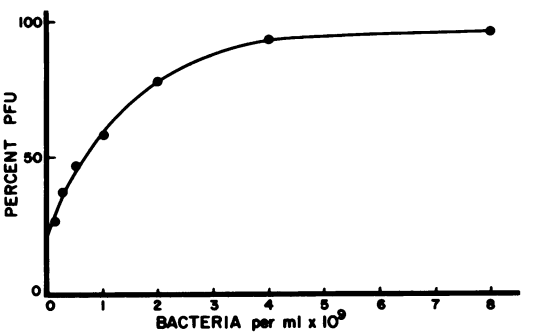

FIG. 2. *Relationship between F pili antiserum (FAB) adsorption and cell concentration. Adsorption mixtures contained 0.25 unit of FAB per ml and HB11 cells as indicated. Per cent plaque-forming units (PFU) equals PFU with FAB after adsorption with cells divided by PFU with control serum (CAB) after adsorption with cells × 100. Each point represents the average of at least three separate experiments.*

Sonic oscillation. An ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., Cleveland, Ohio) was used to rupture cells. A sample of resuspended cells (15 ml) was placed in a 20-ml beaker and chilled to 0 C in an ice bath. The probe was pushed to the bottom of the beaker, and the culture was sonically oscillated for 3 min. With light microscopy, we determined that these preparations consisted mainly of fragments of bacteria with very few whole cells.

Examination of F pili by electron microscopy. In some experiments, the length and number of attached F pili were determined by electron microscopy (18). We examined at least 100 cells for each sample, counted the number of attached F pili, and expressed this as the average number of pili per cell (total number of attached pili divided by the total number of cells examined). The average length of pili in a sample was determined by measuring at least 25 pili which were selected at random. These measurements were not corrected for obscuration of pili by cells (18), since we were interested in relative changes in F piliation. The average number of F pili per cell and the average length of a pilus for each sample are given as percentages of measurements made on control cells.

Preparation of samples for electron microscopy. Several cell cultures that were tested for serum-blocking power were also examined in the electron microscope. Phage R17 (10^{10} PFU/ml) and formaldehyde (final concentration, 3.7%) were added to a sample of culture, and the mixture was incubated at 0 C for at least 10 min to allow for phage adsorption. A drop of this was placed on a copper specimen grid covered with a Formvar film and 30 min was allowed for cells to settle on the grid. The drop was drawn off with filter paper, and the grids were allowed to dry for 20 min to enhance fixation. A drop of 1% phosphotungstic acid adjusted to *p*H 7.0 with NaOH was placed on the grid. After 1.5 min, the stain was drawn off with filter paper until the grid was dry. The samples were then examined in an electron microscope (Phillips EM 300) at a magnification of about 2,800. Areas on each grid were selected at random and photographed.

RESULTS

Inhibition of phage infection by F pili-antiserum. In this study, F pili were assayed by an indirect method involving the ability of FAB to inhibit M13 and R17 phage infection (Fig. 1). At a 1/10 dilution the number of M13 and R17 PFU was reduced 95 and 98%, respectively. The serum was also effective at low concentrations, since a 1/6,400 dilution inhibited infection of both phage by about 20%.

Serum-blocking power of male and female E. coli cells. The ability of several strains of male and female *E. coli* cells to adsorb FAB is shown in Table 1. The FAB concentration in the adsorption mixtures, 0.25 unit per ml, would normally cause a 78% inhibition of M13 infection as shown in Fig. 1. Table 1 shows that the number of PFU obtained after adsorption with female cells was not significantly different from those in nonadsorbed mixtures. This indicates that female cells cannot adsorb FAB. Adsorption with all the male strains tested, on the other hand, resulted in a sevenfold increase in PFU, suggesting that male cells having F pili did adsorb FAB.

Serum-blocking power of HB11. We elected to study F pili on HB11 cells because F pili are the only appendages on these cells visible in electron micrographs. The serum-blocking power of various concentrations of HB11 cells in adsorption mixtures containing 0.25 unit of FAB per ml is shown in Fig. 2. FAB at 0.25 unit per ml was routinely used in subsequent experiments because decreases in FAB due to adsorption would be measurable as a decrease in per cent PFU. Figure 1 shows that the largest change in per cent PFU per unit change in the FAB concentration occurs when the latter is less than 0.5 unit per ml with a lower limit of about 0.01 unit per ml. The curve in Fig. 2 shows that when no cells were present in the adsorption mixture there were 22% PFU, as expected with 0.25 unit of FAB per ml, and that when the cell concentration in the adsorption mixture increased there was an increase in per cent PFU. At 95% PFU, when the cell concentration was 5×10^9 cells per ml, the curve became linear with a gradual slope, indicating that almost all of the input FAB had been adsorbed.

Figure 3 shows the kinetics of FAB adsorption with 4×10^9 cells per ml and 0.25 units of FAB per ml. In this experiment, the cells were added to a mixture containing FAB; at the indicated times, the culture was filtered, and the supernatant fluid assayed for FAB. The bound antibody at each time interval was measured as the difference between the input concentration (0.25 unit per ml) and that remaining in the supernatant fluid after adsorption. The amount of bound FAB increased with time up to about 30 min and then remained constant. The amount of FAB bound at equilibrium by different concentrations of HB11 cells in mixtures containing 0.5, 0.25, 0.125, and 0.0625 units of FAB is shown in Fig. 4. At saturating cell concentrations, the maximum amount of FAB bound seems to be directly proportional to the FAB concentration. One would also expect that bound FAB would be proportional to the cell concentration when FAB was saturating. This may be true; for example, there seems to be a proportional increase in bound FAB at 0.25 unit per ml with cell concentrations less than 5×10^8 cells per ml. However, FAB adsorption at high FAB concentrations could not be determined accurately because our assay measures the difference in FAB concentration before and after adsorption. If FAB is in tremendous excess, this difference would be very

TABLE 1. *Serum-blocking source of various strains of E. coli*

Cells[a]	Strain	Sex	Source	PFU per plate[b]			Per cent plaque-forming units, (A − B)/(C − B) × 100
				(A) +FAB[c] +cells[a]	(B) +FAB −cells	(C) −FAB −cells	
W1895	K-12	Hfr	L. S. Baron	172	29	237	68
200U	K-12	F⁻	L. S. Baron	26	29	237	0
HB11 F⁻[d]	B/r	F⁻		17	29	237	0
SS2	K-12	Hfr	W. S. Knight	140	19	237	59
ERW	K-12	F′/*his*⁺	E. Raizen	150	19	237	65
B380	K-12	F⁻	E. Raizen	21	19	237	1
HB11	B/r	F′/*lac*⁺	H. Boyer	171	25	256	63

[a] Cells were prepared and added to adsorption mixtures (final concentration, 2×10^8 cells/ml).
[b] M13-infected cells, supernatant fluids of adsorption mixtures were the reaction mixtures for M13 infection.
[c] F pili antiserum; the final FAB concentration in each adsorption mixture was 0.25 unit per ml.
[d] HB11 F⁻ is a strain of HB11 cured to F′/*lac*⁺ with acridine orange (10).

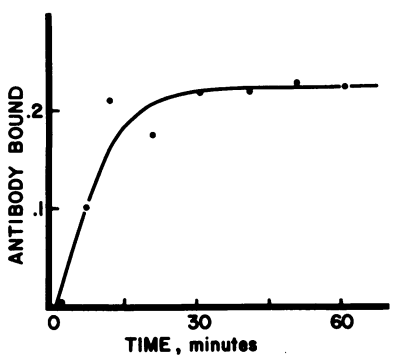

FIG. 3. *Kinetics of F pili antiserum (FAB) adsorption. Adsorption mixtures contained 0.25 unit of FAB and 4 × 10⁸ cells per ml. At the times indicated, samples were filtered through 0.2-μm membrane filters. Antibody bound equals input FAB (0.25 unit per ml) minus the FAB concentrations in the filtrates.*

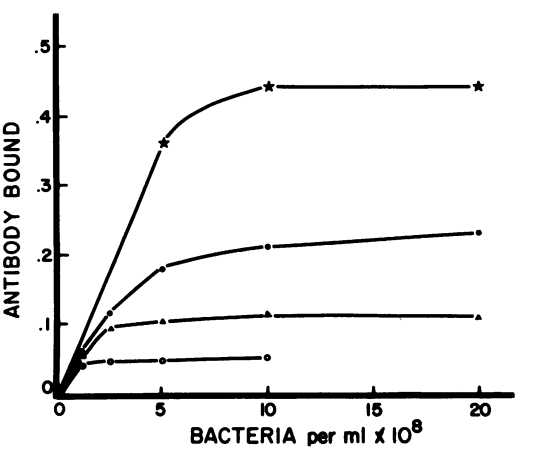

FIG. 4. *Binding of FAB as a function of antibody and cell concentration. Adsorption mixtures contained FAB (in units per ml): 0.5 (★), 0.25 (●), 0.125 (▲), and 0.0625 (○). Cells were added as indicated; 60 min was allowed for adsorption. Bound antibody was determined as in Fig. 3.*

small and essentially unmeasurable.

Effect of blending and sonic oscillation on serum-blocking power. F pili have been removed from the cell surface by blending cultures in various types of homogenizers. Novotny et al. (18) reported a characteristic "blending spectrum" for F pili where the change in F pili per cell, after blending cultures at various speeds, was determined from electron micrographs. We repeated this type of experiment but measured instead the change in serum-blocking power as a function of blending speed. Our results (Fig. 5) indicate that visible F pili and serum-blocking power decrease proportionately, which supports the idea that serum-blocking power is associated with F pili and no other antigen. These results also indicate that the assay is independent of the type of phage used because there was no significant difference between the blending spectrum observed with R17 and that determined with M13. Furthermore, we also observed a similar blending spectrum for serum-blocking power using conjugation as an indicator system (*data not shown*). Of the three indicator systems, R17, M13, and conjugation, which can be used to determine serum-blocking power, we arbitrarily chose M13 for our studies of F pili.

Fragments of F pili, produced by sonic oscillation or by blending, retain their ability to adsorb male phage (5, 26). We sonically treated whole cells and found that sonic oscillation did not de-

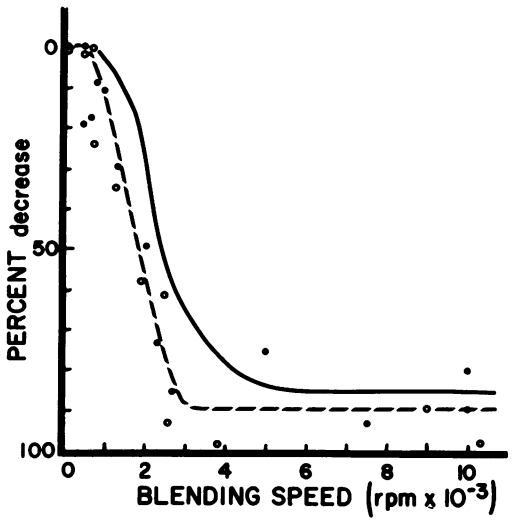

FIG. 5. *Per cent decrease in serum-blocking power and attached F pili as a function of blending speed. The solid line represents previously reported data (18) for the loss in cells with F pili as determined from electron micrographs. The dotted line represents the per cent decrease in M13 (●) and R17 (○) plaque-forming units (PFU) caused by F pili antiserum (FAB) that was not adsorbed by blended cells. Cultures were blended at the indicated speeds, resuspended to 10⁹ cells per ml in Z medium containing 0.25 unit of FAB per ml, and incubated at 0 C for 60 min. Blended cells were removed by centrifugation, and each supernatant fluid was tested for its ability to inhibit either R17 or M13 phage infection. Per cent decrease in PFU equals 100 − 100 × [(PFU adsorption with blended cells minus PFU without adsorption)/(PFU with adsorption by nonblended cells minus PFU without adsorption)].*

TABLE 2. *Effect of blending and sonic oscillation on attached F pili*

Cell treatment	Per cent PFU[a]				Per cent amount of F pili per cell[b]
	Expt 1	Expt 2	Expt 3	Avg	
None	88	83	98	89	100
Sonic oscillation	100	95	115	103	130
Blending	36	27	—	31	4
Blending + sonic oscillation	39	26	—	32	5

[a] Per cent plaque-forming units (PFU) was determined as in Fig. 1.

[b] Per cent amount of F pili per cell equals amount on treated cells divided by the amount on untreated cells × 100.

stroy serum-blocking power and may have even enhanced it (Table 2). An increase in serum-blocking power might be expected if cells contained intracellular F pili since this pool would add to the external pili already present. This possibility was tested by shearing off the external F pili before the cells were sonically oscillated. Because the serum-blocking power of blended, sonically treated cells was not significantly different from the blended cells, we conclude that there are no intracellular F pili capable of interacting with serum. Cells, however, may contain pools of F pili subunits which lack serum-blocking power or they might contain intact pili that are buried in the cell membrane, unavailable to antibody.

Effect of growth temperature on the yield of F pili. Knolle and Ørskov (13) showed that fr plaque yields were maximum when plates were incubated between 34 and 37 C. At lower temperatures the yield declined and reached zero at about 24 C. A number of steps in fr infection might be temperature dependent and responsible for the observed decrease in plaquing efficiency. However, it was also shown that male *E. coli* cells could not adsorb fr if they were grown at 18 C and that they regained their ability to adsorb phage, within an undisclosed period of time, when the growth temperature was shifted to 37 C. It was suggested that pili were not produced by cells at low temperature because the expression of F pili genes on the sex factor was temperature sensitive.

When we measured F pili by serum-blocking power, we found that pili were not present at temperatures below 25 C, but began to appear at higher temperatures, reaching a maximum level at about 37 C (Fig. 6). Since serum-blocking power measures total length or amount of F pili in a sample, there are two interpretations of the data. Either cells produce shorter pili at low growth temperatures or they produce fewer pili, or both. Electron micrographs suggest that the number of cells producing pili decreases. For example, pili produced by cells grown at 30 C were as long, in fact slightly longer, than those on cells grown at 37 C. However, the average number of pili per cell at 30 C was only 36% of the 37 C level. A very few pili, 3% of the 37 C level, were observed on cells grown at 25 C, and there were not enough pili to determine accurately an average length. However, the ones observed fell within the length distribution for 37 C cells.

There could be several reasons for the lack of pili at temperatures below 25 C. Synthesis of pili subunits may not occur at these temperatures, or perhaps the assembly of pili subunits is inhibited. To study this further, we grew cells at 37 C, removed existing pili by blending at 0 C, and resuspended the cells in fresh media adjusted to 37, 30, 25, and 20 C. Shifting the cells down to 0 C and then up to 20 or 37 C did not affect viability. However, growth resumed very slowly,

and there was no appreciable change in cell number or cell mass during the time we studied pili outgrowth (0 to 15 min). F pili production was determined by serum-blocking power (Fig. 7) and, in a separate set of experiments, by electron microscopy (Fig. 8).

The results with serum-blocking power show that new pili were rapidly produced when cells were resuspended at 37 C. Within 5 min, the amount of F pili per cell was about 50% of that for nonblended cells which is consistent with other reports (1, 18, 30). A return to the 50% level could be indicative of complete regeneration if the nonblended cells have two kinds of pili, those in the process of outgrowth and free pili which have adhered to the cell surface (18). The production of new pili at 30 C was slower than at 37 C, and the plateau level reached in 8 min was also lower. It was expected that new pili would be produced at 30 C since cells cultured for several generations at 30 C produce normal size F pili only in fewer numbers (see Fig. 6). At 25 C, the cells produced some pili even though they lack pili when grown at 25 C for several generations (Fig. 6). The increase in pili at 25 C was gradual; however, by 15 min, the amount of pili per cell was 40% of the 37 C level. A temperature of 20 C, on the other hand, seems to inhibit both phases of pili growth. Cells cultured at 20 C do not have pili, and cells grown at 37 C and resuspended at 20 C cannot produce new ones. A simple interpretation of these results is that pili are assembled from a pool of subunits such that temperature can influence either subunit synthesis or assembly, or both. Accordingly, cells grown at 37 and 30 C synthesize pili subunits which form a pool and can be assembled at temperatures as low as 25 C. Cells grown at 25 C, on the other hand, may not synthesize subunits and, therefore, cannot produce pili when grown at 25 C unless a pool is already present from growth at a higher temperature. The inability of cells to make pili when shifted from 37 to 20 C

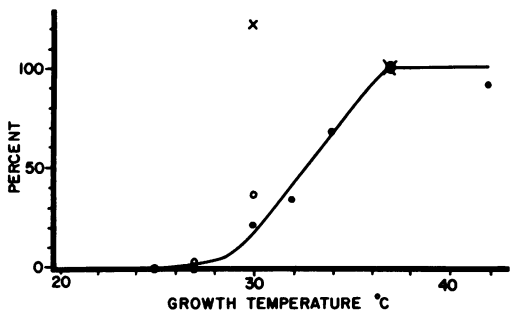

FIG. 6. *Effect of growth temperature on F pili. Cells were grown aerobically for at least 10 generations at temperatures given. Generation times were: 35 min, 37 C; 65 min, 30 C; 115 min, 25 C. Symbols: ●, amount of F pili per cell determined by serum-blocking power; each point represents the mean of at least three experiments; ×, average length of F pili; and ○, the average number of F pili per cell determined by electron microscopy. One hundred per cent equals the values for cells grown at 37 C. At 37 C, the mean ± standard error of two determinations for the average number of pili per cell and average length of a pilus were 0.55 ± 0.07 pili per cell and 2.2 ± 0.1 μm, respectively.*

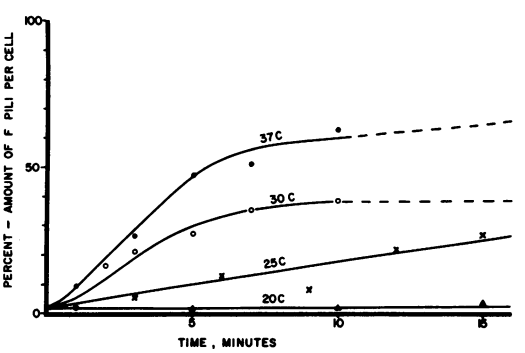

FIG. 7. *Growth of new F pili at different temperatures. Cells were grown at 37 C and blended at 0 C. Pellets were resuspended in fresh media warmed to the temperatures given and incubated aerobically at that temperature. At indicated times, samples were assayed for amount of pili per cell by serum blocking power. Each point is an average of at least three determinations. The amount of F pili per cell for nonblended cells at time zero equals 100%.*

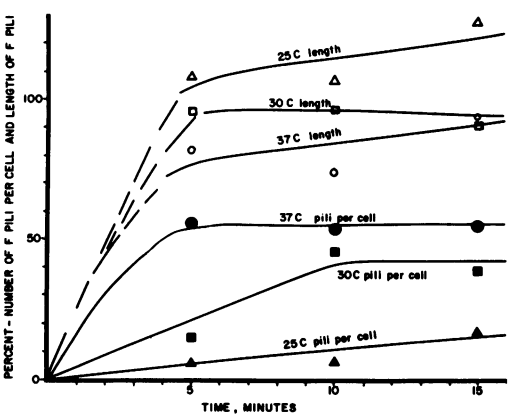

FIG. 8. *Increase in average length of new F pili and the average number of F pili per cell at different temperatures. Experimental conditions were identical to those described for Fig. 7 except values were determined from electron micrographs. F pili per cell: ●, 37 C; ■, 30 C; ▲, 25 C. Average length of F pili: ○, 37 C; □, 30 C; △, 25 C. The average number of F pili per cell and average length of an F pilus on nonblended cells at time zero equals 100%.*

or when grown at 20 C for many generations suggests that both synthesis and assembly have ceased.

Although cells produced pili at 30 and 25 C, the rates were reduced, suggesting that either fewer cells were producing pili or that the pili were shorter because assembly was slower. We examined cells in the electron microscope and found that the primary effect of temperature was to decrease the number of pili per cell, rather than the length of pili (Fig. 8). Cells resuspended at 25 C, for example, had fewer visible pili when compared to cells at 37 C but they were just as long as those produced at 37 C. Similarly, the pili at 30 C were about the same length as pili formed at 37 C, but, again, fewer cells were producing them. A comparison of these kinetics with those obtained from serum-blocking power (Fig. 7) indicates that under these conditions serum-blocking power increases in proportion to number of cells with F pili. The prediction from serum-blocking power studies that the size of the pili produced at 20 and 37 C would have to be the same seems to be borne out by the actual measurements of pili length.

Effect of shifting the growth temperature on F pili production. When cells were grown at 25 C and then shifted to 37 C, they regained their ability to make F pili (Fig. 9). During the first 30 min at 37 C, there was a gradual increase in the amount of F pili per cell. When the growth rate was changed to the usual 37 C rate, the amount of F pili per cell rapidly increased, reaching the 37 C control level by 50 min. The threefold increase in pili that occurred between 40 and 50 min also indicates that pili outgrowth can be very rapid as previous results indicate.

When the growth temperature was shifted *down* from 37 to 30 C and 20 C, there was a rapid loss in amount of F pili per cell that was most pronounced at 20 C (Fig. 10). This result surprised us because the cells cannot make pili at 20 C, and, in the absence of outgrowth, the amount of pili per cell was expected to remain unchanged. In general, there are three ways that these cells could have lost pili in the absence of outgrowth. One is physical breakage which can occur under certain conditions, for example, during blending. However, breakage cannot account for this loss of pili because pili were not lost at 0 C where breakage should still occur. Another possibility is chemical degradation; one can imagine that substances in the culture fluid, enzymes perhaps, degrade F pili, or perhaps pili are just very unstable at temperatures above 20

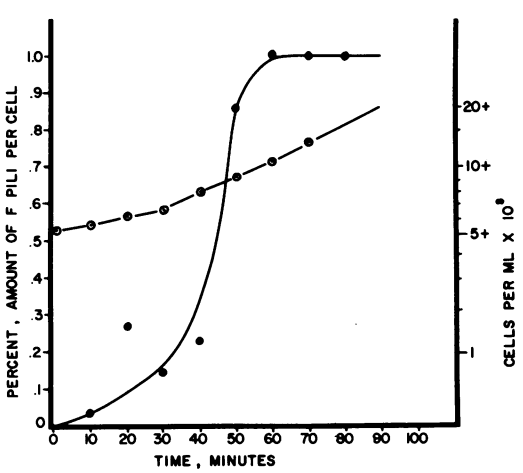

FIG. 9. *Effect of a temperature shift up on the growth of new pili. Cells were grown aerobically for at least 10 generations at 25 C. At time zero the temperature was changed to 37 C, and the amount of F pili per cell (●) at given times was determined by serum-blocking power; O, cell growth. Amount of F pili per cell, on cells grown at 37 C to a concentration of 5 × 10⁸ cells per ml, equals 100%.*

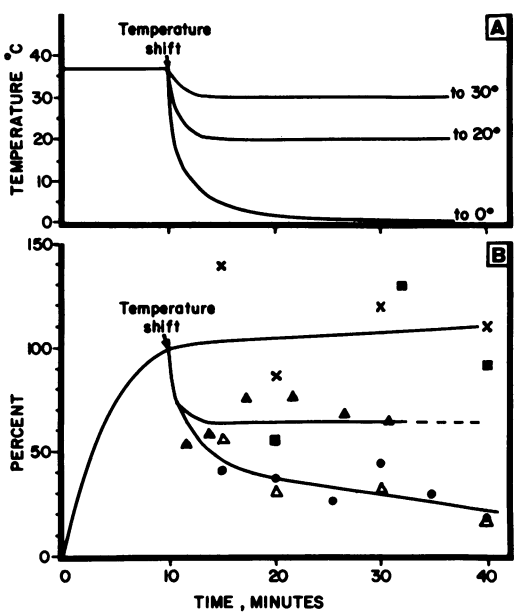

FIG. 10. *Effect of a temperature shift down on F pili production. Cells were grown at 37 C, blended at 0 C, and resuspended in fresh media at 37 C. After 10 min of incubation at 37 C, the temperature was shifted to 30, 20, and 0 C. A, Change in temperature of each mixture as a function of time. B, Amount of F pili per cell at 30 C (▲), 20 C (●), and 0 C (■) as determined by serum-blocking power. The average length of F pili (×) and number of pili per cell (Δ) at 20 C were determined from electron micrographs. One hundred per cent equals either the amount, length, or number of pili for cells at 10 min before the temperature shift.*

C and gradually fall apart. A third possibility is that pili are released from the cell by some process, physical or chemical, that is under cellular control. We examined cells in electron micrographs to determine whether the decrease at 20 C represented a decrease in pili size or a decrease in number of cells with pili. The close correlation between the amount of pili per cell and the number of cells with pili, coupled with length measurements, showing that the average length of pili did not change, suggests that a fraction of the cells had completely lost their pili. This would tend to rule out a degradation process that works on the pilus tip because it should noticeably shorten pili before a reduction in number of cells with pili becomes apparent. A cellular release process, on the other hand, would be consistent with the data if every pilus, regardless of size, had the same probability of being released, and if release occurs at temperatures near 20 C but not at 0 or 37 C.

DISCUSSION

Our results can be divided into two sections pertaining either to serum-blocking power as an assay for F pili or to the influence of temperature on pili growth. In this discussion, we will first consider the advantages and disadvantages of serum-blocking power and other methods for assaying F pili. We will then present a model for the growth of F pili and relate our temperature studies to it.

F pili have been assayed by electron microscopy (1, 2, 9, 14, 18) and by a filtration technique using radioactive, male-specific, RNA phage (3, 11, 28). Electron microscopy provides information on size, number of pili per cell, and therefore, the total amount (total length) of pili attached to cells, but these measurements may be difficult to make accurately. For example, a portion of each pilus is obscured by the cell, so pili appear shorter or may be completely hidden. Cultures also contain free pili that can aggregate end-to-end, readsorb to cells, and overlap cells on microscope grids. All of this can lead to an overestimation of size and the number of pili per cell (18). Although it is possible to see free pili in electron micrographs, no quantitative assay using the electron microscope has been developed.

The phage filtration assay, on the other hand, is fast, easy, and inexpensive (11). It depends on the adsorption of labeled RNA phage along the entire length of pili and, therefore, measures the total amount of pili (total binding sites) in a sample. The filtration assay does not provide any information on size or number of pili per cell. It also assumes that all adsorption sites bind phage equally and that the phage-pili complexes can be trapped on membrane filters. However, the relationship between phage attachment and total amount of pili may not be as simple as first imagined. Phage lysates contain infectious and noninfectious phage which differ in their ability to bind to pili and it may be that certain portions of pili bind phage more strongly than others (3, 24).

In addition, the assay does not detect small pili-phage complexes that pass through filters (26), and there are some conditions where pili no longer bind phage so the assay cannot be used at all. For example, the addition of phenethyl alcohol to a culture prevents phage adsorption to attached pili but not to free pili (27). However, the virtues of phage filtration seem to outweigh its limitations, and the assay is widely used.

We have developed an alternative method for assaying F pili based on serum-blocking power. Since F pili antibody binds along the entire length of pili, this assay, like phage filtration, measures total amount of pili. There are several results which support our conclusion that serum-blocking power measures pili and not some other cellular antigen. It has been shown by others that the F^+ antigen (22) is the F pilus (12, 13) and that F pili antibody binds to the sides of pili (12, 15; C. Brinton, E. Raizen, C. Novotny, and S. Polen, *manuscript in preparation*). No antigen, other than pili, has been shown to be associated with the F^+ donor state. Since purified F pili were used as antigens, it was expected that the antibody produced would be specific for F pili and, therefore, the only specific antibody in this serum capable of inhibiting male phage infection and bacterial conjugation. We showed that only male cells which produced F pili had serum-blocking power and that the loss of visible pili by blending was accompanied by a similar loss of serum-blocking power. Furthermore, the loss of F pili by other treatments and the appearance of new pili on the cell surface was correlated with the disappearance and appearance of serum-blocking power. Serum-blocking power was also correlated with the total amount of pili in a sample confirming the direct observations that antibody molecules bind to the sides of every pilus regardless of its length.

With serum-blocking power, it may be possible to detect small pili that cannot bind phage or are too small to be trapped by membrane filters. The method may also serve as an alternative assay when pili are purposely altered such that they no longer bind phage, but still bind antibody. It may also be possible to take advantage of the specificity of the antigen-antibody reaction and use serum-blocking power to determine the proportions of different F pili subunits present in hybrid

pili (6). Serum-blocking power can be used to study other sex pili since the appropriate antiserum, phage, and conjugation systems have been described (15, 17). Although we used purified F pili as antigens, whole cells or crude pili preparations probably would have worked as well because nonspecific antibody can be removed by adsorption with female cells or blended males. Finally, there might be some advantage to having several detection systems for serum-blocking power. We used infection by phage M13; instead, we could have used infection by an RNA male phage (R17) and probably the formation of mating pairs in bacterial conjugation, since these processes are also inhibited by F pili antibody (C. Brinton et al., Bacteriol. Proc., p. 67).

One major limitation of the method is sensitivity; under our conditions, the minimum cell concentration (pili concentration) that could be detected in absorption mixtures was about 10^8 cells per ml, and best results required 2×10^9 cells per ml. This is about 10 times the concentration required for phage filtration (11). We were able to detect serum-blocking power in culture supernatant fluids, but quantitative measurements of free pili were difficult to make because we could not easily concentrate free pili. It should also be pointed out that serum-blocking power is more difficult to perform than phage filtration, and interpretation of results is accomplished with standard curves that must be accurately determined. However, the standard conditions of our assay were arbitrarily set in many instances, and, therefore, it is likely that the assay could be improved in terms of sensitivity and possibly some experimental procedures could be eliminated entirely.

We also present in this report some temperature studies where we used serum-blocking power to measure the growth of F pili. Before discussing this, we would like to elaborate on a model for the growth of F pili proposed by Brinton (2). The model we favor proposes that F pili are made in the membrane at unique locations (F pili sites) where pili subunits collect and are assembled. Growth begins when an initiation signal triggers the assembly process, and the pilus grows outward as new subunits are added to the base. The subunits may be synthesized at the assembly site or they may migrate to it from other sites of synthesis. When the pilus reaches a certain length, determined perhaps by the size of the subunit pool, it is released from the cell. We imagine that pili release is a consequence of the terminal step in assembly and, therefore, it should not occur under conditions where assembly was inhibited. After release, a new pilus may appear at the vacated site or perhaps at a different site so that the number of pili growing out of a cell at any given time depends on how many sites are actively engaged in making pili. Several things may happen to released pili (free pili); some may join end-to-end to form long pili, others might stick to the cells, and it is possible that free pili are eventually degraded. Accordingly, the level of free pili in a culture would be determined by the rate of release and how fast they are degraded. Both of these processes in turn might be influenced by several factors such as the chemical composition of the media, *p*H, oxygen tension, and temperature.

Our model proposes that pili are assembled in the membrane and in this respect probably resemble some filamentous phage (16). The possibility that pili are made in the cell wall seems unlikely since pili have been seen on spheroplasts (1). Pili do not appear to be in the cytoplasm since we showed that extracts of blended cells did not exhibit serum-blocking power, and S. Polen and C. Brinton (*personal communication*) could not detect internal F pili with a different immunological assay based on complement fixation.

There are more compelling reasons for suggesting that cells have pools of pili subunits. Brinton (1) reported that blended cells made new pili in the presence of chloramphenicol and suggested that there are pools of pili protein. The kinetic studies reported in this paper for pili outgrowth at different temperatures are consistent with this. If outgrowth required protein synthesis or the synthesis of any macromolecule, the rate of outgrowth would be expected to decrease after the temperature is shifted from 37 to 25 C. This is not what we found; when cells were grown at 37 C, blended at 0 C, and resuspended at 25 C, they produced fewer pili but these were as long as those produced at 37 C. This indicates that temperature did not inhibit the rate of outgrowth, but apparently did prevent initiation of outgrowth at some sites. Therefore, it is likely that outgrowth involves the assembly of preexisting subunits, and that assembly has a low temperature coefficient. The ability to produce pili at a nonpermissive temperature such as 25 C also indicates that there is a pool of subunits. We can readily explain pili outgrowth after a shift to 25 C as the assembly of subunits that were synthesized during growth at 37 C. There is, however, no direct evidence that a temperature of 25 C completely inhibits synthesis of subunits, and there may be other reasons for the absence of pili during growth at low temperatures.

Our studies of how temperature influences attached F pili suggest that pili may be released prematurely under some conditions by a mecha-

nism that does not require the assembly of pili subunits. In these experiments, cells were grown at 37 C, blended at 0 C, and allowed to grow new pili for 10 min at 37 C. After 10 min, when the cells had produced about 50% of the pili present before blending, the growth temperature was shifted to 30 and 20 C. At both temperatures some attached pili were rapidly released from the cells. We would expect a loss of F pili at 30 C if the rate of synthesis of subunits decreased and the rate of assembly remained the same because existing pili would grow out and be released from the cell faster than they could be replaced. Eventually a new equilibrium level would be established where the amount of pili per cell would be characteristic for cells growing at 30 C. Our results support this idea, since we found that 60% of the existing pili remained attached to cells at 30 C. This represents 30% of the usual amount at 37 C because we began with cells that had one-half the number of pili at 37 C. This compares favorably with the 20% level of pili found on cells that were grown for several generations at 30 C (Fig. 6 and 10). However, the mechanism of release proposed to explain the loss of pili at 30 C requires assembly and could not account for the loss of F pili at 20 C since we have evidence that assembly does not occur at 20 C (Fig. 6 and 7). Therefore, we must postulate another release process which can operate at 20 C but not at 0 C because no loss was observed at 0 C. The inability of this release process to operate at 0 C might suggest that it requires some metabolic activity. One explanation of this loss is that temperature affects the sites where pili are assembled. For instance, cells grown at 20 C may not produce pili because they cannot produce sites. Pili sites may consist of F pili genes, ribosomes, and membranes, and maintenance of these sites might require macromolecular synthesis and energy production. These processes may be interrupted when cells are shifted to 20 C and contribute to the dissociation of sites and to the release of any pili growing out of these sites. One explanation for why cells have fewer pili at 30 C than at 37 C is that fewer sites can be maintained at 30 C. We are not the only ones to observe a loss of F pili when metabolic activity is restricted. Curtiss et al. (9) reported that cells gradually lost pili when they were starved for essential amino acids, carbon, and energy. Since no loss was observed when metabolic activity was arrested, they suggested that some metabolic activity was necessary for release.

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LITERATURE CITED

1. Brinton, C. C., Jr. 1965. The structure, function, synthesis, and genetic control of pili and a molecular model of DNA and RNA transport in gram negative bacteria. Trans. N.Y. Acad. Sci. **27**:1003-1054.
2. Brinton, C. C., Jr. 1967. Contributions of pili to the specificity of the bacterial surface and a unitary hypothesis of conjugal infectious heredity. *In* B. D. David and L. Warren (ed.), Specificity of cell surfaces. Prentice-Hall, Englewood Cliffs, N.J.
3. Brinton, C. C., Jr., and H. Beer. 1967. The interaction of male-specific bacteriophages with F pili, p. 251-289. *In* J. S. Colter and W. Paranchych (ed.), Molecular biology of Viruses. Academic Press Inc., New York.
4. Brinton, C. C., Jr., P. Gemski, Jr., and J. Carnahan. 1964. A new type of bacterial pilus genetically controlled by the fertility factor of *E. coli* K12 and its role in chromosome transfer. Proc. Nat. Acad. Sci. U.S.A. **52**:776-783.
5. Caro, L. G., and M. Schnös. 1966. The attachment of male-specific bacteriophage f1 to sensitive strains of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **56**:126-132.
6. Cooke, M., E. Meynell, and A. M. Lawn. 1970. Mutant Hfr strains defective in transfer: restoration by F-like and I-like derepressed R factors. Genet. Res. **16**:101-112.
7. Crawford, E. M., and R. F. Gesteland. 1964. The adsorption of bacteriophage R17. Virology **22**:165-167.
8. Curtiss, R. 1969. Bacterial conjugation. Annu. Rev. Microbiol. **23**:69-127.
9. Curtiss, R., L. G. Caro, D. P. Allison, and D. R. Stallions. 1969. Early stages of conjugation in *Escherichia coli*. J. Bacteriol. **100**:1091-1104.
10. Hirota, Y. 1960. The effect of acridine dyes on mating type factors in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **46**:57-64.
11. Ippen, K. A., and R. C. Valentine. 1965. An assay for the male substance (F-pili) of *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **21**:21-27.
12. Ishibashi, M. 1967. F pilus as f^+ antigen. J. Bacteriol. **93**: 379-389.
13. Knolle, P., and I. Ørskov. 1967. The identity of the f^+ antigen and the cellular receptor for the RNA phage fr. Mol. Gen. Genet. **99**:109-114.
14. Lawn, A. M. 1966. Morphological features of the pili associated with R^+F^- and R^-F^+ bacteria. J. Gen. Microbiol. **45**:377-383.
15. Lawn, A. M., E. Meynell, G. G. Meynell, and N. Datta. 1967. Sex pili and the classification of sex factors in enterobacteriaceae. Nature (London) **216**:343-346.
16. Marvin, D. A., and B. Hahn. 1969. Filamentous bacterial viruses. Bacteriol. Rev. **33**:172-209.
17. Meynell, E., G. G. Meynell, and N. Datta. 1968. Phylogenetic relationships of drug resistance factors and other transmissible bacterial plasmids. Bacteriol. Rev. **32**:55-83.
18. Novotny, C., J. Carnahan, and C. C. Brinton, Jr. 1969. Mechanical removal of F pili, type I pili, and flagella from Hfr and RTF donor cells and the kinetics of their reappearance. J. Bacteriol. **98**:1294-1306.
19. Novotny, C., W. S. Knight, and C. C. Brinton, Jr. 1968. Inhibition of bacterial conjugation by ribonucleic acid and deoxyribonucleic acid male-specific bacteriophages. J. Bacteriol. **95**:314-325.
20. Ohtsubo, E. 1970. Transfer-defective mutants of sex factors in *Escherichia coli*. II. Deletion mutants of an F-prime and deletion mapping of cistrons involved in genetic transfer. Genetics **6**:189-197.
21. Ohtsubo, E., Y. Nishimura, and Y. Hirota. 1970. Transfer-

defective mutants of sex factors in *Escherichia coli*. I. Defective mutants and complementation analysis. Genetics **64**:173–188.

22. Ørskov, I., and F. Ørskov. 1960. An antigen termed f+ occurring in F+ *E. coli* strains. Acta Pathol. Microbiol. Scand. **48**:37–46.
23. Ou, J. T., and T. F. Anderson. 1970. Role of pili in bacterial conjugation. J. Bacteriol. **102**:648–654.
24. Paranchych, W., P. M. Krahn, and R. D. Bradley. 1970. Stages in phage R17 infection. Virology **41**:465–473.
25. Trenkner, E., F. Bonhoeffer, and A. Gierer. 1967. The fate of the protein component of bacteriophage fd during infection. Biochem. Biophys. Res. Commun. **28**:932–938.
26. Wendt, L. W., K. A. Ippen, and R. C. Valentine. 1966. General properties of F-pili. Biochem. Biophys. Res. Commun. **23**:375–380.
27. Wendt, L., and H. W. Mobach. 1969. Evidence for two states of F pili. J. Bacteriol. **97**:640–646.
28. Valentine, R. C., P. M. Silverman, K. A. Ippen, and H. Mobach. 1969. The F-pilus of *Escherichia coli*, p. 1–52. *In* A. Rose and J. Wilkinson (ed.), Advances in microbial physiology, vol. 3.
29. Valentine, R. C., and M. Strand. 1965. Complexes of F pili and RNA bacteriophages. Science **148**:511–513.
30. Valentine, R. C., H. Wedel, and K. A. Ippen. 1965. F-pili requirement for RNA bacteriophage adsorption. Biochem. Biophys. Res. Commun. **21**:277–282.