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Mechanical Removal of F Pili, Type I Pili, and Flagella from Hfr and RTF Donor Cells and the Kinetics of Their Reappearance

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The effect of mechanical agitation (blending) on the removal of F pili, type I pili, and flagella from Hfr (high-frequency recombinant) and resistance transfer factor (RTF) fi^+ *Escherichia coli* cells was studied by electron microscopy. The reduction in number and length of appendages was measured as a function of blendor speed under standard conditions of temperature, medium, cell density, and blendor configuration. F pili and flagella were removed within the same narrow range of blendor speeds. Type I pili were removed within a higher and broader range of speeds. The speed which reduced the average length of type I pili to 50% was 3.5 times the speed which reduced the average length of F pili to 50%. None of the speeds employed inhibited cell growth, viability, or the ability to produce cell appendages. The kinetics of reappearance of F pili and type I pili after removal by blending were also different. F pili grew out very rapidly, reaching 50% of their full length in 30 sec and their full length in 4 to 5 min. The number of attached F pili per cell also increased rapidly, reaching a constant value in 4 to 5 min. After 5 min, F pilus lengths were distributed around a modal value of about 1.2 μm, and the numbers of F pili per cell were distributed according to a Poisson distribution, with an average of 1.0 per cell. These reappearance kinetics, length distributions, and number distributions are consistent with a model of F-pilus outgrowth in which new F pili appear at random locations on the cell surface at an average rate of about once every 4 min, grow to their characteristic length in about 4 min, and then separate from the cell. F pili which had separated could absorb to the cells, leading to the presence of two classes of F pili on cells: those in the process of natural outgrowth and those attached by absorption. Type I pili increased in length much more slowly than did F pili, although the fraction of cells having visible type I pili increased very rapidly after blending because of the large number of type I pili per cell. The fraction of flagellated cells increased even more slowly, reaching only 30% of the unblended fraction in 30 min. The application of blending spectra and reappearance kinetics to the identification of cell functions with surface structures is discussed.

Gram-negative bacteria have several types of surface appendages that can be seen in the electron microscope: flagella, F pili, Ib pili, type I-V pili, and other types of pili that have not yet been characterized (2, 10, 17). Flagella are organs of motility (8, 16); F pili and Ib pili (sex pili) have been implicated in the transport of nucleic acid during bacterial conjugation and male-specific phage infection (2, 6, 10), and several possible functions for type I pili have been proposed (2, 7; P. Gemski, Ph.D. Thesis, University of Pittsburgh, Pittsburgh, Pa., 1964).

One of the initial steps in the purification of these surface structures is their removal from cells by some form of mechanical agitation (2, 5, 16). Mechanical removal of structures has also been used to study function. For example, it has been inferred that flagella are organs of motility because the removal of flagella by blending leads to a loss in motility (12, 13). The requirement of sex pili for conjugation and male phage infection has been inferred from the observation that the removal of sex pili by blending results in a loss of ability to form mating pairs or to adsorb and be infected by male phages (2, 6, 10).

However, the blending speeds that were employed in earlier studies were sufficiently high to

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remove all surface appendages and possibly unknown surface substances that could not be seen in the electron microscope. Correlations between removal and loss of function would be more meaningful, and purification procedures would be simplified if blending conditions just sufficient to remove a particular structure could be defined. Preliminary experiments indicated that much less agitation than had been used previously could remove F pili and flagella and that the range of speeds from the beginning of removal to maximal removal was rather narrow. Therefore, we considered it possible to develop a more precise blending technique in which the relationship between the intensity of blending and the removal of a surface structure (the blending spectrum) would be characteristic for each kind of structure. By varying the rotor speed while keeping constant the blending time, blended volume, type of blendor cup, and temperature, we were able to determine that the blending spectra for F pili and flagella were identical, whereas that for type I pili was different.

Another application of the blending technique involves the reappearance of surface structures after their removal. If structures can be removed without inhibiting the ability of the cell to produce new structures, their synthesis and growth can be studied under various physiological conditions. Their function can also be studied by determining the correlation between their reappearance and the reappearance of function. Therefore, we have investigated the kinetics of reappearance of F pili, type I pili, and flagella as well as the effects of blending on cell growth and viability.

The blending spectra and reappearance kinetics of the ability of donor cells to form mating pairs and to adsorb and be infected by deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) male-specific phages are compared with those for F pili in the following paper.

MATERIALS AND METHODS

Bacteria and bacteriophage. Two strains of *Escherichia coli* K-12 were used: W1895 (Hfr Cavalli) and J53-R1-19 (RTF fi^+). The resistance transfer factor (RTF) in J53-R1-19 is a derepressed RTF of the fi^+ type that is transferred at a high frequency and confers resistance to kanamycin (9). Both of these strains have F pili, type I pili, and flagella. W1895 and J53-R1-19 were obtained from L. S. Baron and N. Datta, respectively. We also used bacteriophage M12, which is a male-specific RNA phage that adsorbs to the sides of F pili (2).

Growth conditions and media. Bacteria were grown aerobically at 37 C in Z, Z1, and MGluMet media, as indicated in figure legends. Z and MGluMet media have been described previously (11). Z1 medium is identical to Z, except that it does not contain glucose.

Cell concentrations were determined with a modified electrophotometer (Fisher Scientific Co., Pittsburgh, Pa.) that was previously calibrated in terms of viable cells per milliliter.

Mechanical removal of pili (blending). Cultures of bacteria, growing exponentially in Z, Z1, or MGluMet media at 37 C, were diluted with homologous medium to a cell concentration of 4×10^8 cells per ml. Portions of these cultures (40 ml) were blended for 2 min at one speed in an Omni-Mixer homogenizer (model OM-1150, Ivan Sorvall, Inc., Norwalk, Conn.) with a chamber capacity of 200-ml. Blending speeds, in terms of revolutions per minute, were measured with a Jaquet Precision Speed Indicator (Sorvall). The speeds given in each experiment represent the average of at least five measurements made during the 2-min blending time. Blending was usually performed at 37 C, but in some experiments the cells were chilled to 0 C before blending; formaldehyde was added (final concentration 3.7%), and the cultures were maintained at 0 C during the blending treatment.

Preparation of blended cultures for electron microscopy. After the blendor was turned off (zero-time), the cells were immediately killed with formaldehyde, and the culture was quickly chilled to 0 C. This treatment prevents the outgrowth of pili and flagella. Control cultures (nonblended cells) received identical treatment.

For experiments in which rate of reappearance of a surface structure was studied, the blended culture was incubated aerobically at 37 C immediately after blending. At various times, 1-ml samples of this culture were added to tubes containing formaldehyde, and the tubes were quickly chilled to 0 C.

When F pili were the objects of study, bacteriophage M12 (final concentration 1×10^{10} plaque-forming units (PFU)/ml) were added to each sample to facilitate the identification of F pili. A drop of each sample was then placed on a copper specimen grid covered with a collodion film. After 30 min, the drop of culture was drawn off with filter paper and the grid was washed with a drop of distilled water. About 0.5 min later, the water was drawn off with filter paper until the grids were dry. This method of preparation was chosen in preference to the collodion-agar filtration technique previously used (3) because it caused less clumping of bacteria into large groups. It has the disadvantage of leading to underestimating the number of unattached appendages, since these may not settle on the grid in the time allowed or may be preferentially washed away by the distilled water.

Grids were shadowed with platinum at an angle of 14°. The samples were examined with an RCA EMU-3F electron microscope with an objective aperture of approximately 50 μm; an accelerating voltage of 100 kv was used. A low-magnification pole piece was employed with an initial magnification of approximately 1,800. An area on each grid was selected at random, and micrographs were taken with Kodak electron-image plates. From 20 to 40 cells could be seen in a typical micrograph. The cells shown in Fig. 1 and 2 represent selected areas of micrographs that were enlarged for illustrative purposes.

Examination of surface structures. The effect of

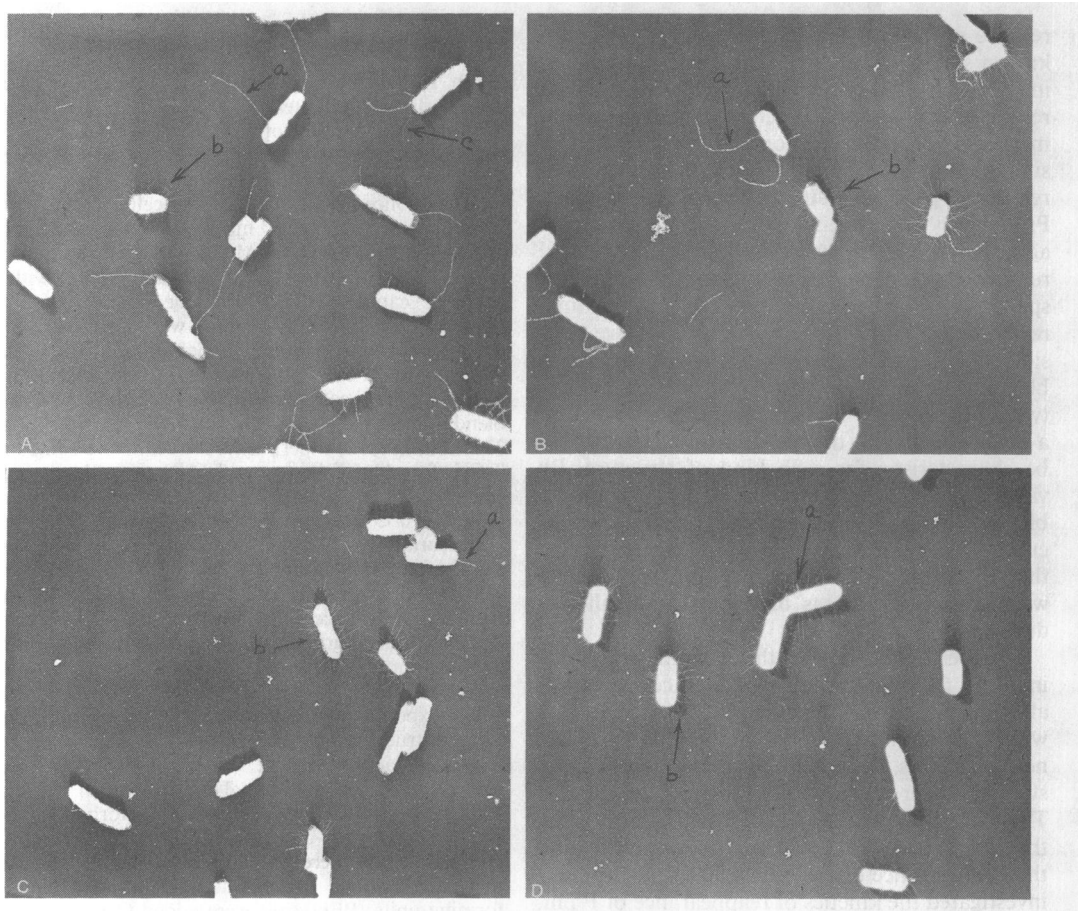

FIG. 1. *Electron micrographs of Hfr cells that were blended in Z medium for 2 min at 37 C. The pictures represent areas of typical electron micrographs selected for illustrative purposes. Cells were blended at the following speeds (rev/min): A, 0; B, 1,050; C, 2,200; and D, 4,200. Symbols: a, F pilus with bacteriophage M12 attached; b, type I pili; c, flagellum.*

blending on cell-surface structures was determined from electron micrographs of blended cells (*see* Fig. 1, 2). Approximately 100 cells from each sample were examined and scored for presence and absence of F pili, type I pili, and flagella. A cell scored as having F pili or flagella had at least one of these structures, of any length, attached to the cell surface. A cell scored as having type I pili had at least 50 or more type I pili, of any length, attached to the cell surface. A cell was scored only if it was reasonably well isolated from other cells, but even this selection method makes scoring for F pili and flagella difficult. These structures can be rather long, and frequently a structure attached to one cell will overlap a neighboring cell. There is also the possibility that free F pili and flagella settle on the microscope grids, and when they overlap the cells they would appear to be attached. Since we could not conveniently control these difficulties, we scored cells as follows. If two cells appeared to be connected by one structure, with no free ends, the structure was assigned to only one of the cells. If two cells appeared to be connected by one structure and one of the cells had an additional structure with a free end, both cells were scored as having the structure. We could not distinguish between structures that had their origin within the cell and free structures that were merely adsorbed to the cell surface.

Length measurements. In experiments in which the removal of pili was studied as a function of blending speed, the lengths of the pili seen in photographic plates were measured with a 10X calibrated eyepiece. For F pili, about 100 cells in each sample were examined, and 15 attached F pili that appeared to be the longest were measured. The length of an F pilus was taken to be an average of these 15 measurements. For type I pili, five type I piliated cells were selected, and five of the type I pili on each of these cells were measured. The length of a type I pilus was taken to be an average of these 25 measurements.

In the experiment in which the reappearance of pili was studied, the lengths of pili seen in enlarged photographs were measured with a ruler. About 150 cells in

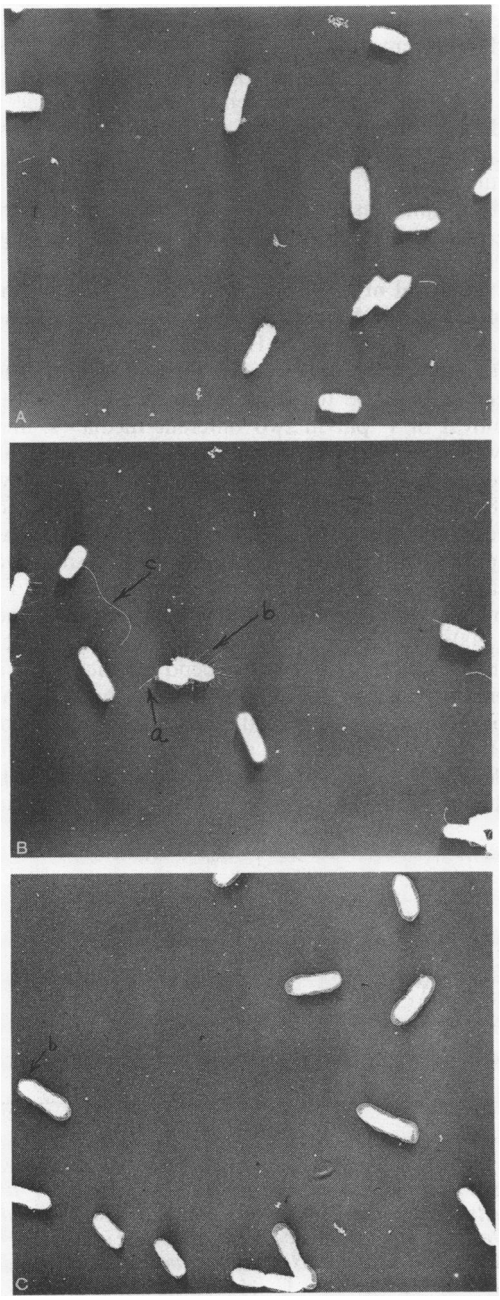

FIG. 2. *Electron micrographs of Hfr and RTF fi+ cells that were blended in Z medium for 2 min at 37 C. Details are as indicated in Fig. 1. Hfr cells (A) were blended at 11,650 rev/min. RTF fi+ cells (B) and (C) were blended at 0 and 9,200 rev/min, respectively.*

each sample were scored for the presence of F pili and type I pili. The protruding lengths of all of the visible attached F pili (usually about 80) were measured. The average protruding lengths of type I pili were determined by measuring the distance between the edge of the cell and the end of the pili fringe. Since type I pili vary in protruding length (0.5 to 2.0 μm), the pili fringe was defined as a line drawn along the outer edge of the dense portion of the fringe.

Corrections for obscuration of appendages by cells in electron micrographs. When appendages are not long compared to the size of the cell, errors can arise in estimating their actual lengths and numbers if only that portion protruding beyond the border of the cell is measured. These conditions apply particularly to the relatively short F pili that reappear immediately after blending. Since our micrographs show only the protruding portion of an appendage, we have devised a method of estimating the actual distribution of appendage lengths from the distribution of protruding lengths. Only the distributions of F pilus lengths, determined as a function of time after blending, and the unblended control distributions have been corrected by this method.

If one assumes that F pili originate at random locations on the cell surface and radiate from these points in straight lines, the distribution of obscured lengths can be determined. This distribution has been determined graphically for a cell with the shape and average size of the cells whose F pilus lengths were measured and is plotted in Fig. 3 for 2-mm intervals (4.65 mm = 1 μm). Most of the obscured lengths are short compared to the cell size (5 by 12 mm), but a few are nearly as long as the cell. It can be seen that the class of F pili whose true length on the photograph is 10 mm will appear as a distribution of protruding lengths, depending on the points of origin and the direction in which the F pili lie. Thus, very few 10-mm pili will be measured as 10 mm long. The distribution of obscured lengths shows that 35% will protrude 8 to 10 mm, 32% 6 to 8 mm, 22% 4 to 6 mm, 7% 2 to 4 mm, 3% 0 to 2 mm, and 1% will be completely obscured. For the class of F pili whose actual length is only 4 mm, 33% will be completely obscured. It can also be seen that a given class of protruding lengths can receive contributions from F pili of different

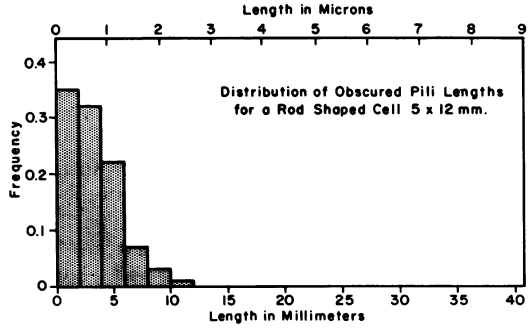

FIG. 3. *Distribution of obscured lengths of appendages for a rod-shaped cell of average size. The distribution was generated graphically by measuring the straight-line distance to the edge of the cell from a large number of points (400), uniformly distributed over the cell, in several directions (8) uniformly distributed around each point.*

actual lengths, since their points of origin may differ. Since a given class of protruding lengths can only receive contributions from F pili of *longer* actual lengths, the actual distribution can be generated from the experimental distribution in the following way. A smooth curve is drawn through the measured distribution of protruding lengths, and the number of pili in each class of protruding lengths is plotted as a histogram with a 2-mm class interval. It is then assumed that the number of pili in the longest protruding class represents 35% of an actual class whose length is equal to the upper limit of that histogram interval. The number of F pili having that actual length is then assumed to be equal to the experimental number of the longest protruding class, divided by 0.35. The contributions to the next five shorter protruding classes are then calculated from the known distribution of obscured lengths and recorded. The next-to-longest protruding class in the experimental distribution is then considered. The contribution to this class from the longest actual length class is subtracted from the experimental frequency. The remaining frequency is then assumed to arise from F pili whose actual length is equal to the upper limit of the next-to-longest class interval. The frequency of F pili having that length is then set equal to the remaining frequency divided by 0.35, and the contributions to the next five shorter protruding classes are added to the contributions to those classes from F pili of the longest actual length. This procedure was iterated (with the aid of a desk-top computer) until an actual length distribution was generated which would give the observed protruding-length distribution.

The average length of an F pilus, the total length of F pili per cell, and the number of F pili per cell were calculated numerically from the actual length distributions. The modal length was taken to be the peak value of the actual length distributions.

RESULTS

Effect of blending on surface structures. Electron micrographs of Hfr and RTF fi$^+$ cells that were not blended or that were blended at various speeds are shown in Fig. 1 and 2. The micrographs of nonblended Hfr and RTF fi$^+$ cells (Fig. 1A and 2B) show that some of the cells have F pili, type I pili, and flagella. The F pili are about 8.5 nm in diameter and about 0.5 to 8 μm in length. Many of the cells pictured have at least one F pilus, and some of them have two or three. A few of the cells are surrounded by a thick fringe of type I pili. These pili are 7.0 nm in diameter and 0.5 to 2 μm in length. Flagella can be distinguished from pili on the basis of size and shape. The flagella on these strains are relatively thick (20 nm in diameter) structures with a uniform wavelength.

A comparison between nonblended Hfr cells (Fig. 1A) and Hfr cells that were blended at 1,050 rev/min (Fig. 1B) shows that there was no significant loss of F pili or type I pili at this speed. A blending speed of 2,200 rev/min (Fig. 2C) was effective in removing some F pili, but this speed did not remove type I pili. At 4,220 rev/min (Fig. 1D), most of the F pili were removed but a few short stubs were present on some cells. This speed did not affect type I pili. F pili and type I pili were completely removed by blending at 11,650 rev/min (Fig. 2A). A similar loss of pili from the RTF fi$^+$ strain was observed when these cells were blended at 9,200 rev/min (cf. Fig. 2B and Fig. 2C).

Removal of F pili and flagella as a function of blending speed. A more precise analysis of the removal of F pili from Hfr and RTF fi$^+$ cells as a function of blending speed is shown in Fig. 4. The curves describe the blending spectra for the removal of F pili in two different media (Z and MGluMet) at two temperatures (37 and 0 C). The results with Hfr cells in Z medium at 37 C show that no significant loss in the fraction of F-piliated cells occurred when the cells were blended at speeds below 1,600 rev/min. However, there was a sharp, continuous decrease in the number of F-piliated cells when the speed was increased beyond 1,600 rev/min. The efficiency of removal under these conditions did not depend on the medium employed (Z or MGluMet) despite the fact that blending causes enriched medium (Z) to foam. However, the apparent efficiency of removal did depend on the physiological state of the cells during the blending process. A sharper decrease in

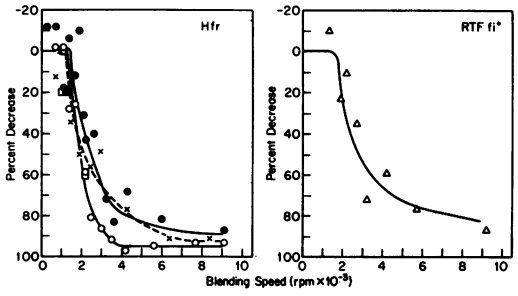

FIG. 4. *Decrease in F-piliated cells and flagellated cells as a function of blending speed. Hfr and RTF fi$^+$ cells were blended for 2 min at the indicated speed. Decrease in F-piliated cells: ●, Hfr cells in Z medium blended at 37 C; ○, Hfr cells in Z medium with formaldehyde, blended at 0 C; ×, Hfr cells in MGluMet medium blended at 37 C; △, RTF fi$^+$ cells in Z medium blended at 37 C. Decrease in flagellated cells: □, Hfr cells in Z1 medium blended at 37 C. Per cent decrease of either F-piliated cells or flagellated cells = 100 × (fraction of F-piliated or flagellated cells in blended mixture/fraction of F-piliated or flagellated cells in the nonblended control culture) − 100. The fractions of F-piliated cells in the Hfr and RTF fi$^+$ control cultures were about 73 and 60%, respectively. The fraction of flagellated cells in the Hfr control culture was 54%.*

F piliation and more complete removal of F pili from Hfr cells was observed when cells in Z medium were chilled to 0 C and killed with formaldehyde before blending. One explanation of this difference is that F pili reappear during blending treatment if the cells are physiologically competent. Some of these "new" pili may be more resistant to blending treatment because they are short. Chilling cells to 0 C and killing them with formaldehyde prior to blending prevents outgrowth; consequently, a greater loss in F-piliated cells would be observed under these conditions. A similar explanation is that F pili grow out very rapidly after the blendor is stopped and they reach an appreciable length before the sample is taken.

RTF fi^+ cells were also blended in Z medium at 37 C. The blending spectrum of their F pili was not significantly different from that for Hfr cells.

The Hfr cultures used to determine the effect of blending on flagellation were grown in Z medium without glucose (Z1 medium). In this medium about 50% of the cells were flagellated versus about 20% when glucose was present. A similar suppression of flagellation by glucose was recently reported by Adler and Templeton (1). A sharp decrease in the number of flagellated cells was observed at blending speeds above 1,500 rev/min. This blending spectrum for the removal of flagella was not significantly different from the blending spectrum for the removal of F pili.

We can imagine several ways in which the removal of F pili occurs. Blending could (i) shear the F pili into smaller pieces, (ii) pull or tear them from the cell at the base in such manner that the F pili remain intact, or (iii) produce a combination of both of these kinds of damage. We found that the loss of F pili was accompanied by a commensurate decrease in the average length of attached F pili (Fig. 5). This suggests that F pili are sheared into pieces at the same speeds at which they are removed since there should be no decrease in the length of those F pili that remain attached to the cell if blending only "pulled" them out. However, it is possible that some pulling or tearing occurs in addition to shearing.

In addition to information about the number and size of F pili that remain attached to the cell, our blending results provide some information about the condition of the removed F pili. Electron micrographs of cultures that were blended at 2,000 to 3,000 rev/min show some pieces of free F pili in the background. These most probably represent F pili that were sheared off the cells. However, at higher blending speeds (9,000 rev/min) we very seldom see pili fragments, which suggests that the majority of free pili are sheared into small pieces that do not settle on the microscope grids or are not visible in our micrographs.

Removal of type I pili as a function of blending speed. The Hfr cells from the preceding blending experiments were also scored for the presence and the absence of type I pili. About 40% of the Hfr cells grown under these experimental conditions have type I pili, and these pili were more resistant than were F pili to blending (Fig. 6). A blending speed of 3,000 rev/min did not cause a significant decrease either in the number of type I-piliated cells or in the average length of type I pili, but the same speed effected a 70% decrease both in the number of F-piliated cells and in the length of attached F pili (*see* Fig. 4). When the blending speed was increased beyond 3,000 rev/min, there

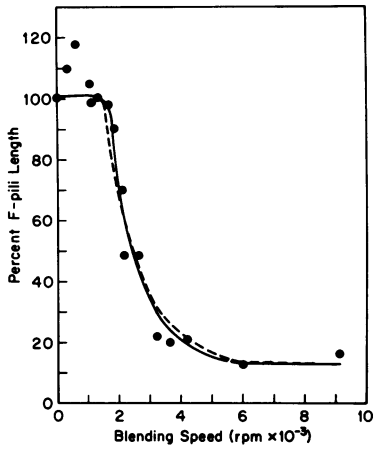

FIG. 5. *Effect of blending on length of attached F pili. Hfr cells in Z medium were blended for 2 min at the indicated blending speed at 37 C. Symbols: ●, per cent length of F pili; broken line, per cent decrease in F-piliated cells (Hfr cells grown in Z medium blended at 37 C), as shown in Fig. 4. Per cent length of F pili = 100 × (average length of F pili on blended cells/average length of F pili on nonblended cells).*

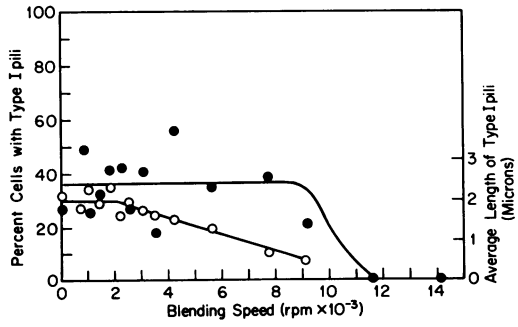

FIG. 6. *Removal of type I pili as a function of blending speed. The Hfr cells in Z medium that were blended at 37 C and scored for F pili (see Fig. 4) were scored for type I pili. Symbols: ●, per cent of cells with type I pili; ○, average length of type I pili.*

was a gradual decrease in the length of type I pili. Since the pili became progressively shorter, there was no decrease in the number of type I-piliated cells until a speed was reached that reduced the length of attached type I pili to the point where they were no longer visible in the electron microscope. This speed was about 11,000 rev/min.

Effect of blending on cell growth and viability. The effect of blending treatment on cell growth and viability is shown in Fig. 7. In these experiments Hfr cells, growing exponentially in Z medium at 37 C, were blended for 2 min at two speeds, 3,400 and 9,500 rev/min. After blending, incubation was continued. Cell mass in each culture, before and after blending, was determined from turbidity measurements, and in one experiment viable counts were made. Cell mass in the blended and nonblended mixtures increased at the same rate, and this increase was accompanied by a proportionate increase in viable cells. These results show that the speeds used to remove pili (1,600 to 9,500 rev/min) do not inhibit cell growth or viability. However, we have found blending conditions in which cell growth is inhibited. A combination of very high blending speeds (greater than 15,000 rev/min) and longer blending times (greator than 2 min) will usually inhibit cell growth. At these speeds and blending times, the heat generated by mechanical work can raise the temperature of the culture above 47 C, a temperature which greatly reduces cell growth. No significant increase in temperature above 37 C occurs when cells are blended for 2 min at 9,500 rev/min with the blended volume and blendor configuration we have used.

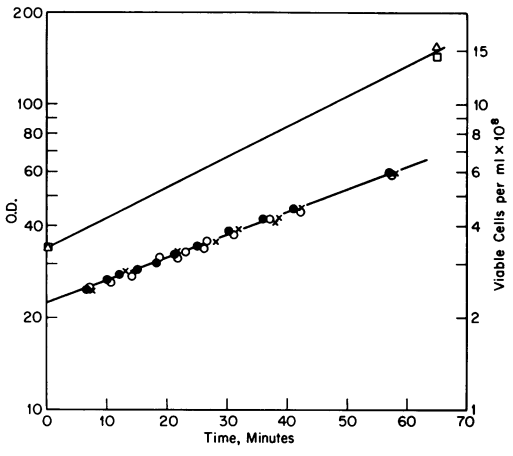

FIG. 7. *Effect of blending on cell growth and viability. For cell growth (viability), a viable cell count on an Hfr culture growing exponentially in Z medium was made at zero-time. The culture was divided into two portions; one portion was blended for 2 min at 9,500 rev/min and then both cultures were incubated aerobically at 37 C. At 65 min, another viable count was made on both the nonblended (△) and blended (□) cultures. For cell growth (mass increase), an Hfr culture growing exponentially in Z medium was divided into three equal portions (zero-time) that were incubated aerobically at 37 C. Turbidity measurements (optical density) were made at the indicated times. At 10 min, one portion was blended for 2 min at 3,365 rev/min (●). At 13.5 min, another portion was blended for 2 min at 9,787 rev/min (○). (×), nonblended cells.*

Reappearance of F pili, type I pili, and flagella. We measured the reappearance of pili and flagella by blending Hfr cells in Z medium for 2 min at 9,500 rev/min, and then we incubated the culture aerobically at 37 C. At various times after blending, further reappearance of surface structures was stopped by chilling portions of the culture to 0 C and adding formaldehyde. These samples were then examined in the electron microscope.

Enlarged prints of random fields were made, and the fractions of cells with each kind of appendage were determined. The protruding lengths of all F pili and the distance from the edge of the cell to the edge of the type I pili fringe were measured. The distributions of actual F pili lengths (protruding lengths corrected for obscuration) at various times after the blendor was stopped, and also for unblended portions of the same culture at the beginning and at the end of the experiment, are shown in Fig. 8.

The distribution of F-pilus lengths in an unblended culture is rather broad, ranging from 0.5 to about 10 μm. The distribution is skewed towards longer lengths around a modal value of 1.7 μm. The distribution of the same unblended culture one generation time (30.5 min) later is not significantly different, indicating that these distributions represent the equilibrium situation in an unblended culture.

Removal of all F pili by blending has a very marked effect on the length distribution. (That all F pili were actually removed during blending was demonstrated by examining a culture which had been blended while cold in the presence of formaldehyde to prevent growth of new pili during the blending process.) The distribution 0.5 min after blending is much narrower than that of the unblended culture, and the modal length is shorter. The distribution broadens and the modal length increases until about 4 min. After 4 min, there is little significant change in the distribution other than the appearance at 25 and 30 min of a few more F pili in the 5-to-10-μm range. Even after one generation time (30 min), the blended distribution has not returned to the unblended distribution, which has many more longer F pili, although the modal length is almost the same.

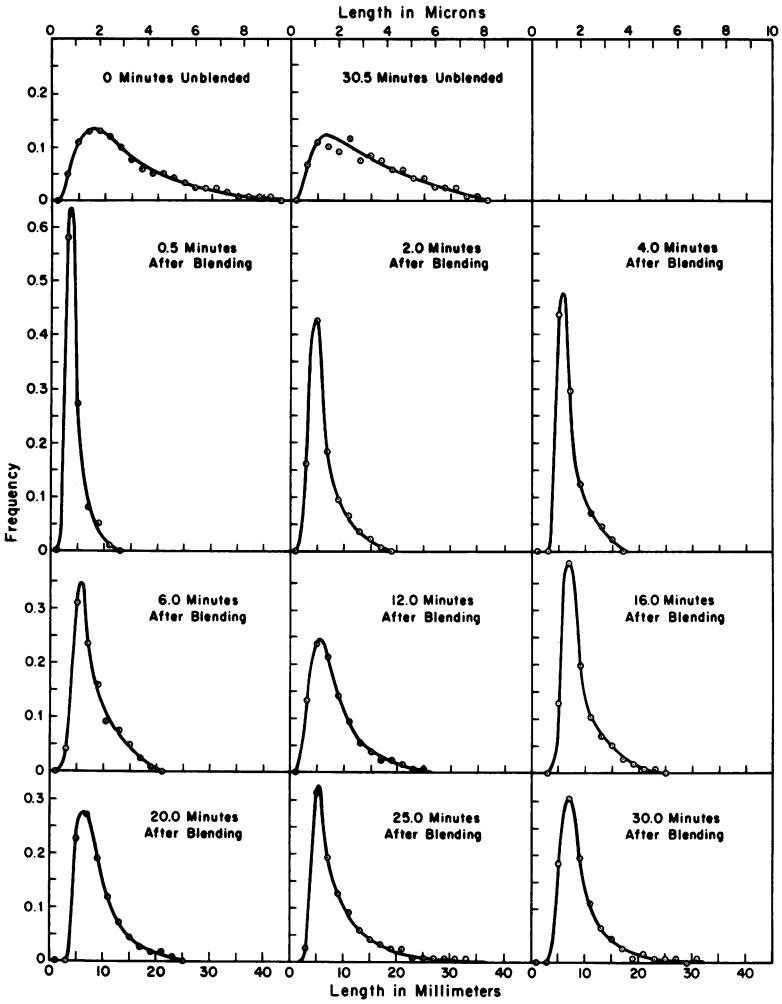

FIG. 8. *Distributions of F-pilus lengths corrected for obscuration, as a function of time after blending. Log-phase Hfr cells (W1895) were blended for 2 min at 9,500 rev/min in Z medium. At various times after blending, the cultures were chilled and fixed with formaldehyde. F-pilus lengths were measured from enlarged electron micrographs.*

At this point, it seems pertinent to consider possible reasons for the failure of the blended culture to achieve the unblended distribution of F-pili lengths. Since the blending process itself had no effect on the viability or growth rate of the culture and F pili having the same modal length as in the unblended culture reappeared on the cells very rapidly, it is unlikely that the distribution was different because of some difference in cell physiology induced by blending. A much more likely explanation is that two classes of F pili, indistinguishable in electron micrographs, appear to be attached to the cells. One class is naturally attached and is in the process of outgrowth, and the other class represents formerly free F pili which have reattached by adsorption to the cell surface or by end-to-end aggregation with naturally attached F pili. The adsorption of free F pili to cells was suspected for two reasons. (i) F pili longer than those seen on cells are always observed in concentrated pure preparations, indicating end-to-end aggregation such as that which occurs in tobacco mosaic virus and DNA male phage preparations. (ii) The few bacteria remaining in partially concentrated preparations of F pili often appear to have 20 to 40 F pili, a condition which is never observed in the starting culture. Therefore, we performed an experiment to ascertain whether adsorption of free F pili existing in the unblended culture was sufficient to account for the increased fraction of long F pili observed in the unblended cultures. A culture of W1895

(HfrC) was grown and blended at 9,500 rev/min in the same way as in the reappearance experiment. To one sample of blended cells was added an equal volume of fresh medium, and to the other sample was added an equal volume of a supernatant fluid from the unblended culture which contained microscopically visible, free F pili. After 30 min of incubation, samples were prepared for electron microscopy and length distributions of F pili were determined. The distributions shown in Fig. 9 clearly show that in the presence of added free F pili the distribution of attached pili is skewed towards longer pili, although the modal length is only slightly longer. In addition, the following parameters, which can be calculated from the data given in Fig. 9, were all lower in the blended culture which had no added free F pili than they were in the culture which had them: number of F pili per cell, 65%; total length of F pili per cell, 47%; number of F-piliated bacteria, 72% (assuming that F pili are distributed among cells according to a Poisson distribution). Therefore, we conclude that the greater proportion of long F pili, the larger number of F pili per cell, and the larger total length of F pili per cell in the unblended cultures compared to the plateau level in the blended cultures arise from those free F pili previously synthesized and separated from the cells which reattach by adsorption to the cell surface or to the ends of naturally attached F pili.

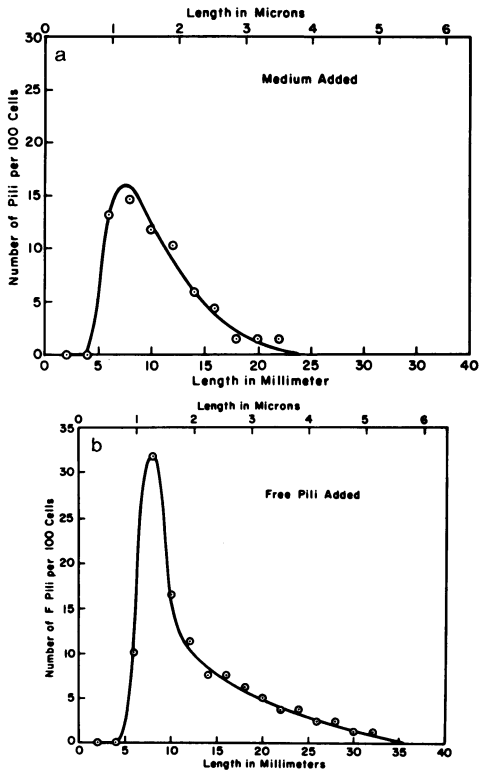

FIG. 9. *Effect of adding free F pili on the length distribution of F pili. Log-phase Hfr cells (W1895) were blended for 2 min at 9,500 rev/min in Z medium and divided into two parts. An equal volume of fresh medium was added to one part (a) and an equal volume of culture supernatant fluid was added to the other part (b). After 30 min, the cultures were chilled, formaldehyde was added, and the distributions of F pilus lengths were determined.*

Some of the parameters of F-pilus outgrowth which are of interest are (i) their characteristic length, (ii), the rate of length increase of an individual growing F pilus, (iii) the average number of F pili per cell, (iv) the rate of appearance of new F pili, (v) the rate of separation of F pili from the cell, (vi) the distribution of F pili over the surface of an individual cell, (vii) the distribution of F pili among cells, (viii) the total amount of F pilus material per cell, (ix) the amount of free F pilus material per cell in the culture, and (x) the characteristic length of free F pili.

An estimate of all of these parameters, except the amount (ix) and characteristic length of free F pili (x), can be obtained from our results.

The characteristic length of naturally attached F pili can be obtained from the plateau-level length distributions in blended cultures in which adsorption of free F pili is negligible. The narrowness of the length distribution for the earlier times after blending (Fig. 8) and the constancy of the peak value (modal value) in the distributions for 4 min or longer after blending indicates that F pili actually have a characteristic length. The rate of increase of the modal value after blending is plotted in Fig. 10. It can be seen that a modal length of 1.2 to 1.3 μm was reached at about 4 min and remained constant for the rest of one generation time. Of this characteristic length, 60% was

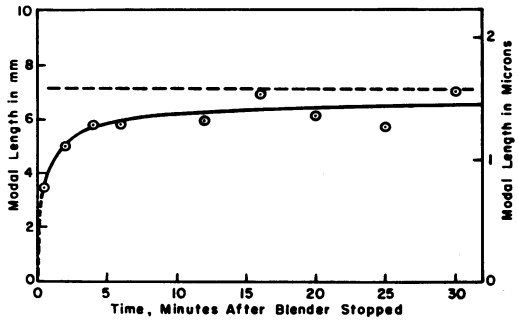

FIG. 10. *Increase of modal length as a function of time after blending. Modal lengths were measured from the corrected distributions in Fig. 8. The broken line represents the modal length of the unblended culture.*

reached in 30 sec, indicating that when a new F pilus appears it grows out very rapidly. The modal length in the unblended control cultures was only a little higher (1.5 to 1.6 μm) and remained constant throughout the experiment.

The average number of F pili per cell is plotted as percentage of the control-culture number for various times after blending (Fig. 11). The number of F pili per cell increases rapidly and reaches a constant level equal to 71% of the control culture at about 4 to 6 min. It was surprising at first that the number of F pili per cell did not return to the level of the control culture. However, the additional number of F pili per cell in the control culture could be entirely accounted for by adsorption of free F pili to the cell, as shown by the addition of an unblended supernatant fluid, containing free F pili. In this experiment, the number of F pili per cell without added free F pili was 65% of the number with added free F pili. Therefore, we conclude that the number of naturally attached growing F pili returns completely to the level of the unblended control culture in 4 to 6 min.

The average total length of F pili per cell is plotted as percentage of the control culture value for various times after blending (Fig. 12). Again, the total amount of F pili increases rapidly, reaching a constant level at 4 to 6 min. The percentage of the control level that is reached is even lower than that for the number of F pili per cell because the free F pili that adsorb to unblended cells in the control culture increase the average F pilus length more than they do the average number of F pili. The reconstitution experiment described above showed that after 30 min the culture without added free F pili had only 47% of the total length of F pili per cell compared to the culture with added free F pili. Therefore, we conclude that the total length per cell of naturally attached growing F pili also returns completely to the level of the control culture in 4 to 6 min.

The increase in the fraction of F-piliated bacteria compared to the unblended culture is plotted in Fig. 13. The fraction has been corrected for obscuration by assuming that those F pili that were completely obscured were also distributed among the cells according to a Poisson distribution. The fraction of F-piliated bacteria could then be estimated as the value $1-e^{-m}$, where m is the average number of F pili per cell calculated from the corrected length distributions. The kinetics of increase of F-piliated bacteria are essentially the same as those for the increase in number (Fig. 12) and total length of F pili (Fig. 13), with somewhat different zero-time and plateau levels. The plateau level of 82% is in good agreement with the previously described reconstitution experiment in which the number of F-piliated bacteria in the blended culture without added free F pili was 72% of that in the blended culture with

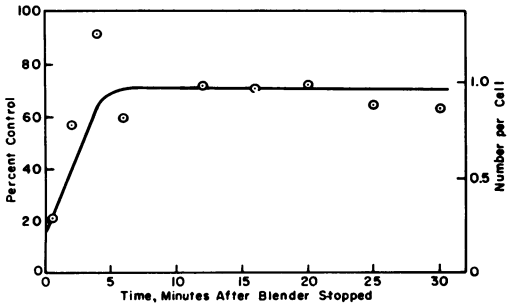

FIG. 11. *Increase of number of F pili per cell as a function of time after blending. The number of F pili was determined from the corrected distributions in Fig. 8. The number of F pili per cell in the unblended control culture is represented by 100%.*

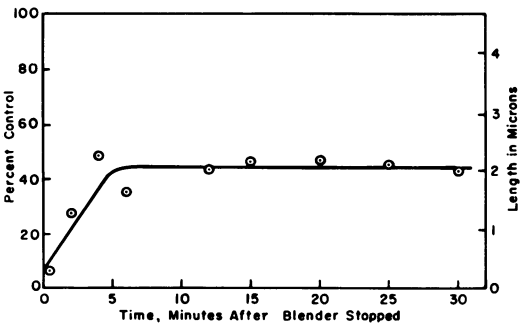

FIG. 12. *Increase of total F-pilus length per cell as a function of time after blending. The total length was determined from the corrected distributions in Fig. 8. The total length of F pili per cell in the unblended control culture is represented by 100%.*

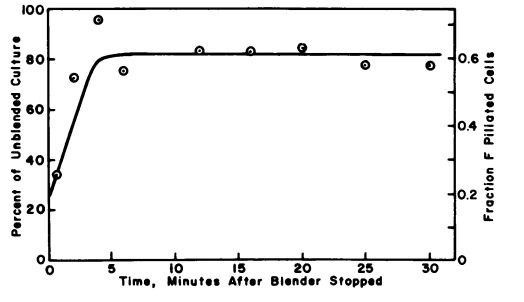

FIG. 13. *Increase in the fraction of F-piliated cells as a function of time after blending. The fraction of F-piliated cells was taken to be $1-e^{-m}$, where m is the average number of F pili per cell determined from the corrected distributions in Fig. 8. The fraction of F-piliated cells in the unblended control culture is represented by 100%.*

free F pili added. We concluded that the number of bacteria with naturally attached F pili reaches the control level in 4 to 6 min.

The distribution of F pili among cells is, in all cases, not significantly different from a Poisson distribution. This is consistent with any mode of F-pilus outgrowth in which the probability of an F pilus appearing is constant with time. Since we do not observe any preferred point of origin, the probability of appearance is probably equal for all points on the surface of the cell.

The increase in the uncorrected number of type I-piliated cells and the increase in the protruding fringe length of type I pili as a function of time after blending are shown in Fig. 14. No corrections for obscurement were made since type I pili are much more numerous than F pili and the fringe length was considered an adequate indicator of type I pili reappearance. Type I pili, like F pili, are not completely removed by blending at the speed used here (9,500 rev/min; *see* Fig. 6), and they were thus about 0.2 μm long when the earliest measurement was made at 30 sec. The increase in *number* of type I-piliated cells, which is reduced by blending to only about 50% of the control, was very rapid because of the large number of type I pili per cell; it returned to the control level in 1 to 4 min.

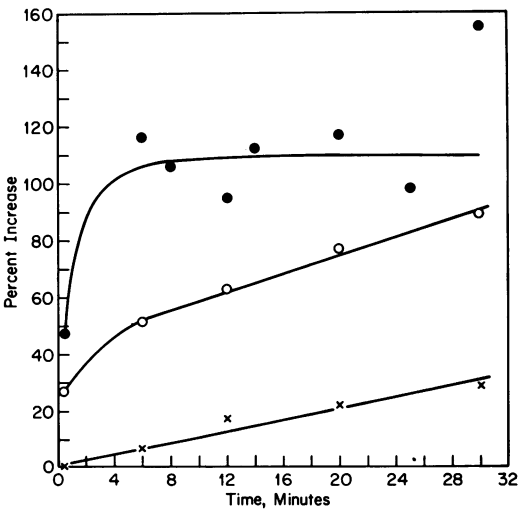

FIG. 14. *Reappearance of type I-piliated cells and flagellated cells and increase in length of type I pili for the Hfr cells described in Fig. 8. The average length of the type I pili was measured from the cell boundary. Measurements were compared to those obtained with nonblended cells (100%). Symbols: ●, per cent increase in fraction of type I-piliated cells; ○, per cent increase in average length of type I pili; ×, per cent increase in fraction of cells with flagella. Fractions of type I-piliated cells and flagellated cells in the control culture were 31.4 and 18%, respectively. Average length of type I pili in the control culture was 0.48 μm.*

The fringe length of type I pili increased from 35 to 90% of the control length in 30 min. This corresponds to a linear-outgrowth rate of about 0.017 μm per min. This is to be compared with a rate of outgrowth for F pili of about 1 μm per min, a rate 60 times as fast.

Flagella, which were completely removed by blending at 9,500 rev/min, reappeared very slowly. The fraction of flagellated cells (Fig. 14) increased at a constant rate, reaching approximately 30% of the unblended culture in 30 min. The increase in the average length of flagella could not be accurately determined because there were very few flagella to measure. Also, the gradual increase in the fraction of flagellated cells indicates that cells began to produce new flagella at different times. Consequently, variation in length would be expected solely on the basis of delays in the initiation of flagella outgrowth. These findings are consistent with other reports that flagella regenerate very slowly (13, 14).

DISCUSSION

Our experiments show that a range of blending speeds, extending from the beginning of removal to complete removal, is characteristic for each surface appendage. The curve for the reduction in length of an appendage versus blendor speed we shall call the "blending spectrum for length." The curve for removal of an appendage versus blendor speed we shall call the "blending spectrum for removal." Blending spectra may be concisely described either by the speed that reduces the length of appendage by 50% or that removes 50% of the appendages or by the width of a speed range between two arbitrarily chosen fractions of length reduction or removal (for instance, the 20 to 80% range).

The blending spectra for removal of F pili and flagella were identical and had a mid-point for removal of 2,200 rev/min and a 20 to 80% range of 1,500 to 3,500 rev/min. The blending spectrum for length of F pili was not significantly different from that for removal. We did not determine the blending spectrum for length of flagella. Type I pili were more resistant to blending than were either F pili or flagella. The blending spectrum for length of type I pili had a mid-point of 7,000 rev/min and a range of 4,000 to 9,500 rev/min.

Cell growth (mass increase) and viability were not affected at the blending speeds we employed, and blending spectra seemed to be independent of the medium in which blending took place. Cell density and time of blending might be expected to influence the degree of removal, but they were not

studied here and were kept constant. The blendor configuration (volume and shape of cup; shape, size, number, and position of rotor blades; and volume of culture blended) was shown in preliminary experiments to markedly affect efficiency of removal; therefore, it also was kept constant.

The blending-spectrum technique should make correlations between function and structure more meaningful than did previous methods in which blending was performed at a single, high speed. If a structure is both necessary and sufficient for a function, its blending spectrum should be the same as that of the function. If the blending spectra for structure and function are different, the structure may not be related to the function or it may be one of two or more structures, all of which are required for the function. In the latter instance, the blending spectrum for function would be the same as the blending spectrum for that required structure which was removed at the lowest blending speeds.

If the blending spectra for structure and function are identical, either the structure is required for the function or the observed structure has the same blending spectrum as another structure which is actually required for the function but which may be inapparent in electron micrographs. Our findings that flagella and F pili have the same blending spectrum for removal and that type I pili have a different one may be taken as an estimate of the reliability of structure-function correlations with this method. Most structures probably have different blending spectra, but caution must be exercised in interpreting such results since differences in sensitivity to blending arise from mechanical properties which are relatively nonspecific.

The kinetics of reappearance of surface structures after removal by blending can also be used to study function. Function should return with the same kinetics as does a structure if the structure is both necessary and sufficient for function. When a function reappears more quickly than a structure, one may conclude that the structure is not necessary for that function. Alternatively, when a structure reappears before a function, it means that the structure is either unrelated to that function or that blending removes or inactivates another structure which is required for the function and which reappears more slowly. This structure may or may not be detectable by electron microscopy. There is also the possibility that a function and an unrelated structure will return at the same rate by coincidence. However, coincidence is much more unlikely with reappearance kinetics than with blending spectra, since differences in the kinetics of reappearance depend upon differences in synthesis, assembly, and outgrowth of structures which are likely to depend upon cell physiology in different ways for different structures. Therefore, if a structure reappears at the same rate as a function, it is, barring an unlikely coincidence, required for that function. One cannot exclude the possibility that an additional structure which would be removed by blending at a higher speed is also required for the same function. Neither can one exclude the possibility that a structure removed at a lower speed but which reappears more quickly than the observed structure is also required for the function.

A further complicating factor inherent in the interpretation of structure-function correlations is that structures may have microscopically inapparent differences induced by blending which affect the function in question. For example, an appendage may need to be completely intact in order to function at all. It would be impossible to distinguish in electron micrographs between an intact appendage and a fractured one. In this case, the reappearance of function might appear to be slower than the reappearance of the appendage if an appreciable number of nonfunctional fractured appendages continued to grow out. Knowledge of blending spectra can also be useful in developing purification procedures for surface structures. The initial step in the purification of pili, flagella, and the K88 antigen of *E. coli* usually involves the removal of these structures from the cell by blending (2, 14, 16). If the minimal amount of agitation necessary to remove a structure is known, contamination with other surface structures, such as type I pili in an F-pili preparation, could possibly be avoided. In addition, unwanted structures removable at lower blendor speeds could be removed beforehand by preblending cells at low speeds.

The size of appendages recovered after blending may also be important. Our finding that the blending spectra for length and removal of F pili were identical suggests that F pili are sheared into smaller fragments by about the same speeds that remove them from cells. This observation is of importance in filtration assays in which the amount of free F pili is determined indirectly by the binding of radioactive male phage to pili (2, 4, 15) and by retention of the F pili-phage complexes by a filter. If high speed blending were employed, the pili fragments would be relatively small and some of these might pass through the filter. C. Brinton and H. Beer (4) and W. Paranchych and R. Danziger (*personal communication*) have found that the male-phage adsorption capacity, measured by the filtration assay, of supernatant fluids of blended Hfr cultures decreases when they are blended at high speeds. This suggests that the F pili were sheared into small

pieces that passed through filters or that the F pili were affected in some other way so that the phage could no longer attach to them.

Our studies of F-pilus outgrowth confirm previous reports from this laboratory (2, 3, 4, 6) that they reappear very rapidly after blending. The kinetics of their reappearance suggest that the mode of outgrowth of F pili is quite different from that of type I pili and flagella. Whereas type I pili and flagella are constantly increasing in length throughout the generation time of a cell, F pili reach a constant length and a constant number per cell in about one-seventh of a generation time. One possible explanation of this rapid return is that blending somehow triggers the outgrowth of F pili. However, the most probable explanation is that F pili are rapidly appearing on the cell surface and just as rapidly disappearing from it. If the F pili remained on the cell surface, the number of F pili per cell and the fraction of F-piliated bacteria would continue to increase throughout the experiment, rather than reaching a constant value at 4 min. F pili could disappear from the cell surface by growing out to a characteristic length and separating from the cell, growing out to a random length and breaking off when they became long enough to be sensitive to shear forces, growing out and then retracting back into the cell, or by growing out and solubilizing into the medium. The facts that F pili have a characteristic length, are found in large numbers free in the culture medium, and are mechanically removable only by an amount of agitation much greater than that used to grow cultures lead us to propose that F pili grow out to a characteristic length and then separate from the cell. This mode of outgrowth is similar to that of the DNA male phages and is consistent with a virus-like structure for F pili previously suggested (2, 3, 6). The characteristic length of the F pilus could possibly be determined by a nucleic acid molecule of fixed length.

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