AN ASSAY FOR THE MALE SUBSTANCE (F-PILI) OF ESCHERICHIA COLI K-12*

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Crawford and Gesteland (1964) have recently described a new type of bacterial appendage. These workers observed, in electron micrographs, that RNA bacteriophages adsorbed to long filaments on the surface of male cells of E. coli K-12. Female cells did not synthesize the filaments and were resistant to infection by the virus. Brinton, Gemski, and Carnahan (1964) have named these thin filaments F-pili, to distinguish them from other more common forms of pili (fimbriae) synthesized by both male and female strains. Male bacteria had from 0-4 F-pili per cell which could become covered along their entire length by phage added at a high multiplicity. Brinton et al. (1964) also demonstrated that F-pili are genetically controlled by the fertility factor of E. coli K-12. These interesting findings stimulated us to try to devise an in vitro assay for the male substance or F - pili synthesized by these strains. Electron micrographs (Valentine and Strand, 1965) helped to confirm the idea that the thin, filamentous F-pili and the "male substance", measured by the filtration assay described below, are identical, and we use these terms interchangeably. In this communication we describe an improved assay procedure for F-pili and show that these filaments are synthesized by a variety of male strains of E. coli and related bacterial strains which have become infected with the fertility factor of E. coli K-12.

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Results and Experimental

The bacteria and bacteriophage strains and general experimental conditions were described earlier (Valentine and Strand, 1965). Free F-pili, as well as F-pili attached to cells were assayed using a modification of the filtration method (Valentine and Strand, 1965). A sample of free F-pili, suspended in Tryptone-Yeast Extract-Calcium broth (TYE) (Loeb and Zinder, 1961), or of a suitable male culture, grown at 37° in this broth to approximately 2 x 10^8 cells per ml, was added to a test tube containing TYE broth, normally to a final volume of 3 ml, though this volume varied with certain experiments. A 0.1 ml volume of P^{32} labelled phage (usually 2 x 10^8 viable phage particles per ml of assay mixture; approximately 12,000 CPM per ml assay mixture) was then added. After 5 min adsorption time at 0°C (or a suitable interval) the samples were quickly diluted to stop further interaction and filtered to remove unadsorbed phage. Radioactive particles which had become attached to F-pili remained on the filter pad and were washed and counted as before (Valentine and Strand, 1965). The assay appears to measure only simple phage adsorption to F-pili, subsequent "injection stages" being prevented by the incubation at 0°, while formation of the F-pili-phage complex occurs readily at this temperature. The filtration assay, then, provides a measure of the quantity of F-pili synthesized by a bacterial culture.

For all experiments reported here, TYE broth replaced the Tris-Calcium assay mixture used earlier. This change was found to be desirable because of the high background levels observed for several preparations of radioactive phage when using the Tris-Calcium mixture. Spontaneous precipitation or aggregation of radioactive phage appeared to be caused by an excess of salt in the assay mixtures. Calcium ion (0.003 M), normally found stimulating for the formation of phage-F-pili complexes, caused spontaneous precipitation of one purified bacteriophage preparation. Likewise, addition of zinc and copper ions (0.005 M), as well as other metals, resulted in a

high amount of radioactivity on the filter, presumably due to the aggregation of the phage particles by the metal. Small amounts of anti-phage serum caused similar effects. No spontaneous precipitation has been observed with six different radioactive phage preparations when TYE broth was used in the assay mixture, and we assume that such aggregation is prevented in the broth, although the reason for this is unknown.

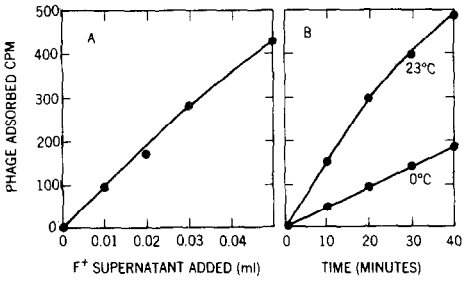

Fig. 1A. Phage adsorption as a function of F-pili concentration in cell-free F^+ culture supernatant. E. coli (K 38) grown to cell density of about 5 x 10^8 cells/ml in TYE broth at 37°C. Cells removed by centrifugation. F-pili assayed by filtration method as in text except reaction volume was 1 ml and incubation was for 30 min at 20°C.

Fig. 1B. Rates of Phage Adsorption to F-pili at different temperatures. Cell-free supernatant (0.05 ml) and reaction conditions as for 1A except incubation volume was increased to 8 ml. 1 ml aliquots were assayed at times and temperatures indicated.

The values obtained by the filtration assay, usually expressed as CPM phage adsorbed per ml of sample, are relative ones since it has not been possible to determine the absolute number or titer of F-pili in a preparation (see Valentine and Strand, 1965, for further discussion of this problem). Also, since the chemical nature of F-pili is not known, a specific unit of F-pili activity has not been developed. Clearly, it would be desirable to have a more standard expression for F-pili concentration; however, the present method was found to be completely adequate for the comparison of relative concentration of F-pili in different preparations.

The data in Fig. 1A demonstrate the high sensitivity obtainable with the improved assay method. F-pili could be detected in as little as 0.01 ml of the spent culture broth of a male strain after the cells had been removed by centrifugation. As will be shown in a later experiment, F-pili are readily sheared from male cells, even during normal growth, explaining their presence in the supernatant. It should be noted that the assay sensitivity shown was attained by a prolonged incubation time. The data in Fig. 1A also show that phage adsorption to F-pili present in the cell-free supernatants was proportional to the quantity of culture broth added over a range of 0 to 0.05 ml of broth.

As described earlier (Valentine and Strand, 1965), the rate of phage adsorption to F-pili is relatively insensitive to temperature and takes place readily even when incubation is at 0°C. The effect of the incubation temperature on the formation of F-pili-phage complexes is shown in Fig. 1B. As seen from Fig. 1B, the rate of adsorption at 0°C was appreciable and was about 35 per cent the rate at 23°C. Increasing the incubation temperature to 37°C had little effect. The rapid rate of phage adsorption occurring at 0°C was of great advantage in measuring attached F-pili in later experiments since at this temperature subsequent injection stages

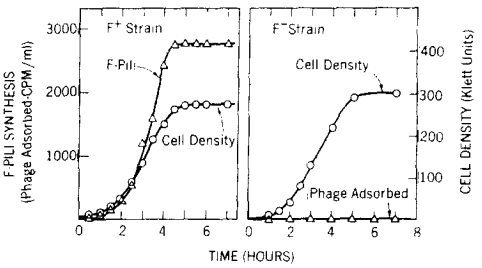

Fig. 2 (Male). Synthesis of F-pili by an E. coli F^+ strain. E. coli K 19 (F^+) grown in TYE broth at 37°C; appropriate aliquots assayed for F-pili as described in text.

Fig. 2 (Female). Control culture E. coli K 17 (F^-), the female parent of K 19, shows no production of F-pili.

were totally prevented; also, additional cellular synthesis of F-pili ceased making possible an accurate measure of F-pili in a culture.

As can be seen in Fig. 2, F-pili synthesis parallels the growth of a culture of male (F^+) cells. Although apparent in detectable quantities during the lag phase, pili synthesis increased most rapidly during the exponential phase of cell growth, reaching a plateau during the early stationary phase, and remaining at a relatively constant level for at least 24 hrs; the later points are not included on the graph. This observation again illustrates the stability of F-pili and indicates their resistance to destruction by cellular enzymes during this period. A large number of free F-pili as well as those still attached to the cells are, as indicated previously, included in the total observed.

As expected, at no time during the growth of the female (F^-) culture did any evidence of phage adsorption appear. Any radioactivity observed, therefore, appears to be attributable to background amounts of nonspecific adsorption of radioactive phage to the cellulose nitrate filter or a certain amount of "trapping" of phage by the cells during filtration. All male cultures, on the contrary, however different in their total pili production, showed a definite dependence of phage adsorption to cell concentration, although the exact period of cell division, during which pili biosynthesis was most active, could not be determined with such randomly dividing cultures.

A number of male (F^+, F', and Hfr) and female (F^-) bacterial strains were tested by the filtration assay for F-pili. These results, summarized in Table I, show that all male strains tested synthesized F-pili, whereas female strains did not. For this experiment all cultures were grown to a density of approximately 2×10^8 cells/ml in TYE broth at 37°; in certain cases where individual strains multiplied slowly, less dense cultures were used for the assay, and the values obtained were corrected to account for the density of the culture. The column labelled "Whole Culture" in Table I

refers to the total phage adsorbed (as P^{32} phage) to 1.0 ml of bacterial culture after 5 min incubation at 0°C. Total assay volume was 3 ml. A bacteriophage to cell ratio of approximately 1 was used in the assay; this corresponded to about 11,000 CPM if 100 per cent of the viable phage had adsorbed. The "Whole Culture" column then refers to phage adsorbed to both free F-pili fragments and F-pili still attached to the cell. The values obtained are, as mentioned, relative ones. The highest values were obtained with male E. coli K-12 strains.

TABLE I

Synthesis of F-Pili by Fertile Strains of E. coli and Related Strains. Assay conditions as in text.

Bacterial Strain	F Property	Source	Quantity of F-pili in Terms of CPM P^{32} Phage Adsorbed	
			Whole Culture/ml	Supernatant/ml
Escherichia coli K-12 (K 38)	F^+	N. D. Zinder	850	428
E. coli K-12 (1028)	Hfr	A. J. Clarke	806	538
E. coli K-12 (1485)	F^+	A. J. Clarke	579	399
E. coli K-12 (3000)	Hfr	A. J. Clarke	600	424
E. coli K-12 (K 19)	F^+	N. D. Zinder	767	614
E. coli K-12 (K 17)	F^-	N. D. Zinder	0	3
E. coli K-12 (2Δ6)	F^-	C. D. Willson	10	0
E. coli B	F'	H. B. Boyer	378	191
Salmonella typhimurium (SR 279)	F^+	N. D. Zinder	80	10
Salmonella typhimurium (SR 318)	Hfr	N. D. Zinder	183	45
Shigella flexneri (69)	Hfr	S. Falkow	216	48
Shigella flexneri (K 60)	F'lac	N. D. Zinder	38	-
Proteus mirabilis	F'lac	C. D. Willson	84	17
Proteus vulgaris	F^-	C. D. Willson	5	0

It was of interest to assay the relative levels of free F-pili fragments in cell-free supernatants of various strains. The column in Table I labelled "Supernatant" summarizes these data. The cell-free supernatants were prepared by centrifuging samples of the various cultures at 12,000 x g for 10 min. Samples of 1.0 ml of the cell-free supernatants were assayed in the manner given above for the cell culture. Again the values were somewhat variable; in the case of several E. coli strains as many as 50 per cent of the F-pili were present in the "Supernatant" whereas with Shigella Hfr (69) only 25 per cent were free.

In general the data of Table I agree with the electron microscopic observations of Brinton et al., 1964, who observed F-pili in several preparations of grown negative bacteria including E. coli, Salmonella, Shigella, Serratia, and Proteus. That F-pili may be synthesized even by various Caulobacter strains, was indicated by the electron micrographs of Schmidt and Stanier, 1965.

It appears likely that the simple assay for F-pili described here will be useful for elucidating their role in phage infection and conjugation. The chemical nature of F-pili is also of considerable interest. The filtration assay has allowed us to obtain highly concentrated F-pili preparations from cell-free supernatants and their purification is presently under way; as yet, however, little is known about their composition. One of the most interesting questions remaining is whether or not F-pili-nucleic acid complexes are "intermediates" in phage infection and conjugation.

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