Effects of Temperature and Energy Inhibitors on Complex Formation between *Escherichia coli* Male Cells and Filamentous Phage fd

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The effect of temperature and various energy inhibitors on the formation of a complex between *Escherichia coli* male cells and filamentous phage fd was studied by a novel filtration method. Centrifuged male cells were observed by electron microscopy to have lost the majority of pili and to produce complexes with fd only above 25 °C. After preincubation of the cells at 37 °C without addition of the phage, nearly half the level of complex formation observed at 37 °C was detected at 0 °C. Complex formation at various temperatures (0 to 37 °C) between cells preincubated at 37 °C and fd was at a minimum at about 20 °C. Several energy inhibitors and uncouplers drastically reduced complex formation at 37 °C, and also at 0 °C if the cells were briefly exposed to the reagents at the end of preincubation. Alteration of the cellular ATP concentration, either by shift-down of temperature or by the addition of the reagents, accompanied alteration in the ability of cells to form a complex with fd as well as alteration of the number of pili on the cell surface. In contrast to earlier reports, these results indicate that the complex formation between male cells and filamentous phage does not proceed either when pili disappear from the cell surface because of a decrease in the cellular energy level or when pili are removed by mechanical forces. The results also show that phage fd adsorption itself is not energy-dependent.

INTRODUCTION

Two major groups of phage are known whose receptor sites are F plasmid-specified pili of *Escherichia coli*: these are spherical RNA phages such as MS2, f2 and Q β and filamentous DNA phages such as fd, f1 and M13. The former phages adsorb to the sides of the pili (Brinton, 1971), whereas the latter adsorb to the tips (Caro & Schnös, 1966). The early steps of infection by these filamentous phages still remain unclear. For example, there have been several different reports on the effect of temperature and cyanide on complex formation with the filamentous phages. Tzagoloff & Pratt (1964) showed that the binding of M13 to male cells proceeded at 0 °C or in the presence of KCN, and Novotny & Fives-Taylor (1974, 1978) reported that cells exposed to NaCN or a temperature of 50 °C adsorbed M13 in spite of the disappearance of pili. In contrast, Marco *et al.* (1974) reported complete abolition of M13 adsorption upon exposure of the cells to 0 °C or KCN and they considered that M13 adsorption required active metabolism. Similar results were also reported by Smilowitz (1974) for f1 adsorption.

One of the reasons for these discrepancies seems to be the lack of a reliable assay system for the phage–bacterium complex, since the pili are extremely fragile. To solve this problem we developed a filtration assay for the formation of the filamentous phage–bacterium complex (Kanegasaki *et al.*, 1978). This made it possible to study precisely the early step of filamentous phage infection, without exposing the phage–bacterium complex to disruption by centrifugation and resuspension. The use of a filtration assay for complex formation with

a filamentous phage has so far been restricted by high background levels of radioactivity on the nitrocellulose filters without male cells. This difficulty has now been eliminated by employing cellulose acetate filters.

Differences in the cell suspensions prepared by different investigators may also have led to the different results. As we report in this paper, different physiological states of cells give different results. We therefore employed centrifuged cells grown at 37 °C in a rich medium. Such cells have lost the majority of their functional pili by centrifugation but pili can be recovered by a short incubation. In this report we show the effects of temperature and various energy inhibitors on the formation of phage fd-male cell complexes using such cells. The results clarify a point of confusion in the earlier literature.

METHODS

Bacteria and phage. *Escherichia coli* K12 strain YC233 (a *met*⁺ derivative of W1895, Hfr derived from Hfr Cavalli) and strain CR34 (F⁻) were used as the male cell and female control, respectively. The standard bacterial suspensions were prepared as follows. One ml of overnight culture in L-broth (Lennox, 1955) without glucose was inoculated into 80 ml of the same medium and the cells were allowed to grow at 37 °C with vigorous shaking. When the absorbance reached 0.6 (at 600 nm in a Shimadzu-Bausch & Lomb spectrometer, usually equivalent to 1.2×10^9 cells ml⁻¹), shaking was stopped and incubation was continued further for 1 h. Cells were harvested by centrifugation at 8000 g for 10 min at 4 °C and resuspended in one-third of the original volume of pre-cooled fresh L-broth. Final suspensions contained 3.6×10^8 cells ml⁻¹. In this paper, we describe these cells as 'centrifuged cells' to distinguish them from 'preincubated cells'. The former had lost the majority of their pili and the latter were fully pilated.

Phage fd was kindly provided by Dr I. Watanabe of Keio University, Tokyo, Japan. For the measurement of F pili, *Escherichia coli* K12 strain JE3100 (*fla*, *pil*, F⁸⁺), kindly provided by Dr Tomoeda of Eisai Co. Ltd, Tokyo, was used.

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazine (CCCP; Sigma), sodium cyanide (Nakarai), thenoyltrifluoroacetone (TTFA; Merck) and sodium azide (Koso Chem.) were used. Firefly lantern extract and ATP were obtained from Calbiochem.

Preparation of ³⁵S-labelled phage. This was made by the method of Kanegasaki *et al.* (1978). Cells grown in LSTG medium (Hershey, 1955) were infected with fd. To 30 ml of this culture, 5 mCi of carrier-free H₂³⁵SO₄ was added 4 h after fd infection. Phages concentrated by partial dehydration in a dialysis tube were purified two or three times by banding in a stepwise gradient (30 to 50 %, w/v) of CsCl in ϕ 80 buffer (Baldwin *et al.*, 1966). Centrifugation was at 48000 rev. min⁻¹ in a Spinco SW 50.1 rotor for 3.5 h.

Filtration assay of the complex between male cells and phage fd. This was done as described by Kanegasaki *et al.* (1978). Centrifuged cells (0.1 ml) of strain YC233 prepared as described above (containing 3.6×10^8 cells) were mixed with 0.1 ml of chilled L-broth, followed by preincubation at 37 °C, if required. ³⁵S-labelled phage fd (20 μ l, 8600 c.p.m., 6.7×10^4 plaque-forming units c.p.m.⁻¹ in a fresh preparation) was added and the mixture was incubated under the conditions indicated in the figure legends. It was then filtered through a Gelman Metrical GA6 filter (pore size 0.45 μ m, 25 mm diam., cellulose acetate) and the filter was washed with 4 ml of ice-cold Tris/HCl buffer (30 mM, pH 7.4) supplemented with 0.85 % NaCl. Radioactivity retained on the filter was counted using Bray's scintillation fluid.

Measurement of F pili by electron microscopy. A suspension of strain JE3100 cells was prepared in the same way as described above for YC233. Then 0.1 ml of this suspension was mixed with 0.1 ml of L-broth and preincubated at 37 °C for 30 min. Inhibitors were added at 37 °C and incubation was continued for 5 min, or the mixture was shifted to 25 °C (without inhibitor) and incubated for 10 min. Samples were fixed with 1.5 % (v/v) formaldehyde solution and dropped on to 200 mesh grids which were covered with collodion and coated with carbon. For negative staining, 4 % (w/v) uranyl acetate was used. The pili (unaggregated) were counted under the electron microscope (JEM 100B).

Assay of cellular ATP concentration. The cellular ATP concentration was measured by a luciferin-luciferase assay (Lundin & Thore, 1975), using an ATP photometer (SAI Technology Co., San Diego, U.S.A.). Firefly extract (50 mg) was dissolved in 20 ml of 0.1 % bovine serum albumin solution containing 10 mM-MgSO₄ and 1 mM-EDTA. The pH was adjusted to about 7.4 with KOH. This enzyme solution was used after storage in a refrigerator for 10 h to eliminate the endogenous luminescence. To extract ATP, 0.5 ml of the bacterial suspension (9×10^8 cells) was added to 2 ml of boiling buffer (20 mM-Tris/HCl, pH 7.8, containing 2 mM-EDTA) and the mixture was boiled for 90 s. The assay was performed by adding 0.5 ml of the boiled solution to 0.5 ml of the enzyme preparation.

Table 1. *Number of F pili per cell after various treatments*

Centrifuged cells of strain JE3100, a mutant lacking flagella and common pili and carrying an F plasmid, were preincubated at 37 °C for 30 min or kept in ice (Incubation 1) and then incubated further under the conditions shown (Incubation 2). The number of F pili was determined electron microscopically as described in Methods.

Incubation 1	Incubation 2	No. of F pili	No. of cells	No. of pili per cell
None	None	52	289	0·18
37 °C, 30 min	None	42	39	1·07
37 °C, 30 min	NaCN (5 mM), 37 °C, 5 min	38	108	0·35
37 °C, 30 min	NaN ₃ (50 mM), 37 °C, 5 min	22	109	0·20
37 °C, 30 min	CCCP (50 µM), 37 °C, 5 min	48	163	0·29
37 °C, 30 min	Arsenate (10 mM), 37 °C, 5 min	34	176	0·19
37 °C, 30 min	25 °C, 10 min	37	77	0·48

CCCP, Carbonyl cyanide *m*-chlorophenylhydrazine.

RESULTS

Examination of the basic experimental conditions

An Hfr culture was chilled, centrifuged and resuspended, and the resulting suspension (described as centrifuged cells) was used to assay complex formation with fd by the quick filtration method. Up to 30% of ³⁵S radioactivity of fd was retained on the filter after incubation for 20 min at 37 °C, but almost no activity was detected if fd was added at 0 °C. However, when the centrifuged cells were preincubated at 37 °C (designated as preincubated cells) before addition of ³⁵S-labelled phage fd, a considerable amount of complex formation was observed even at 0 °C (Fig. 1). This occurred when male but not female cells were used, and was slightly inhibited by chloramphenicol in the reaction mixture.

The number of pili per cell for centrifuged and preincubated cells was 0·18 and 1·07, respectively (Table 1). The ability of preincubated cells to form a complex with fd at 0 °C was abolished by re-centrifugation at 4 °C (Fig. 1). These results indicate that centrifuged cells have lost the majority of functional pili on the cell surface, and that the pili can be recovered by re-incubation at 37 °C. The results also show that the complex formation itself proceeds even at 0 °C as long as functional pili are available.

Effects of temperature shift on fd complex formation with centrifuged and preincubated cells

Complex formation between fd and centrifuged male cells was assayed at various temperatures (Fig. 2). Below 20 °C, little complex formation occurred. Above 25 °C more complex was formed with increasing temperature up to 37 °C. In contrast, if preincubated cells were shifted to temperatures between 0 and 37 °C, complex formation was at a minimum at about 20 °C and increased above or below this temperature (Fig. 3). The effect of a temperature shift to 46 °C or 50 °C is shown in Fig. 4. When preincubated cells were shifted to 50 °C for 3 min, complex formation at 0 °C was completely abolished.

Effects of energy inhibitors and uncouplers on complex formation by centrifuged and preincubated cells

Complex formation between fd and centrifuged cells at 37 °C was strikingly inhibited by energy inhibitors and uncouplers such as NaCN, CCCP, arsenate (Fig. 5), TTFA, NaN₃ and dinitrophenol. The inhibition was not reversed by the addition of glucose, suggesting that the energy required is not supplied by the formation of ATP by glycolysis. Inhibition by arsenate was not reversed by D-lactate, and so the energy generated by the lactate system (Larsen *et al.*, 1974) seems not to be involved. Complex formation at 0 °C by preincubated cells was also completely abolished if they were exposed for 3 min to these agents at 37 °C

Fig. 1

Fig. 2

Fig. 1. Complex formation at 0 °C between phage fd and preincubated cells. Centrifuged cells were preincubated at 37 °C in L-broth for 0 min (●), 5 min (▼), 10 min (△) or 30 min (▲). After cooling the tubes in an ice-water bath, ^{35}S -labelled phage fd was added at 0 °C and complex formation at 0 °C was assayed after 5, 10 and 15 min incubation. For comparison, complex formation at 37 °C (○), complex formation at 0 °C after re-centrifugation of cells preincubated at 37 °C for 30 min (□) and complex formation at 0 °C between fd and female cells preincubated at 37 °C for 30 min (■) are shown. Complex formation is expressed as the percentage of phage retained on the filter. (Radioactivity added to the reaction mixture=100 %.)

Fig. 2. Effect of temperature on complex formation between phage fd and centrifuged cells. ^{35}S -labelled phage fd was added to the centrifuged cells which had been kept for 3 min at 37 °C (○), 33 °C (▼), 30 °C (■), 27 °C (▲), 25 °C (△), 20 °C (□) or 0 °C (●), and the mixture was incubated for the period indicated. Complex formation was assayed as described in the Methods.

Fig. 3

Fig. 4

Fig. 3. Effect of temperature shift on complex formation between phage fd and preincubated cells. Cells preincubated at 37 °C for 10 min were transferred to the indicated temperatures. ^{35}S -labelled phage fd was added 3 min after transfer. Complex formation was assayed, as described in Methods, after 20 min incubation at the indicated temperature.

Fig. 4. Effect of temperature shift on complex formation at 0 °C. Cells preincubated at 37 °C for 10 min were incubated at 50 °C (□), 46 °C (▽) or 0 °C (▲) for 3 min and then chilled in ice. After 3 min at 0 °C, ^{35}S -labelled phage fd was added and complex formation at 0 °C was assayed as described in Methods. For comparison, complex formation at 37 °C (○) or 0 °C (●) between centrifuged cells and ^{35}S -labelled phage fd are shown.

Fig. 5. Effect of energy inhibitors added at 37 °C on complex formation between phage fd and centrifuged cells. The inhibitors were added to suspensions of centrifuged cells of strain YC233 followed by phage. Complex formation was assayed after incubation at 37 °C for the indicated periods: ●, inhibitor present; ○, inhibitor absent. Inhibitors were (a) 5 mM-NaCN, (b) 50 μM-CCCP and (c) 10 mM-arsenate.

Fig. 6. Cellular ATP concentrations at various temperatures. Centrifuged cells of strain YC233 (1.8×10^9 cells in 1 ml L-broth) were preincubated for 10 min at 37 °C and then the temperature was shifted to that indicated. After incubation for 20 min, cellular ATP concentrations were assayed; results are expressed as a percentage of the ATP concentration at 0 °C.

before fd addition, but these agents did not affect the extent of complex formation if they were added at 0 °C.

Effects of temperature shift and energy inhibitors on the cellular ATP concentration

Since the formation of phage-male cell complexes was strongly inhibited by energy inhibitors, we measured cellular ATP concentrations at various temperatures or in the presence of inhibitors, after preincubation at 37 °C (Fig. 6). The cellular ATP concentration was lowest at around 25 °C and increased above or below this temperature. The ATP concentration was significantly decreased by the addition of energy inhibitors or uncouplers to preincubated cells at 37 °C (Table 2). Below 37 °C the ATP concentration seemed to correlate with the extent of complex formation. When the preincubated cells were shifted to 50 °C, the ATP concentration was increased to rather more than twice that at 37 °C (results not shown).

Table 2. *Decrease of cellular ATP concentration in the presence of inhibitors*

Centrifuged cells of strain YC233 were preincubated at 37 °C for 10 min or kept in ice (Incubation 1) and then treated with the agents indicated (Incubation 2). Cellular ATP concentrations were assayed as described in Methods.

Incubation 1	Incubation 2	ATP concn (nmol per 1.8×10^8 cells)*
None	None	300
37 °C, 10 min	None	350
37 °C, 10 min	NaCN (5 mM), 37 °C, 5 min	180
37 °C, 10 min	TTFA (1 mM), 37 °C, 5 min	110
37 °C, 10 min	CCCP (50 μ M), 37 °C, 5 min	180
37 °C, 10 min	Arsenate (10 mM), 37 °C, 5 min	10

TTFA, Thenoyltrifluoroacetone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

* The background counts given by the enzyme solution alone (0.5 ml firefly extract) were usually equivalent to less than 1.3 nmol ATP.

DISCUSSION

We have shown that complex formation between centrifuged male cells and phage fd proceeded only at temperatures above 25 °C. Below 20 °C, practically no complex was formed. In contrast, cells preincubated at 37 °C gained the ability to form a complex with the phage even at 0 °C. After such preincubation the number of pili reached approximately one per cell. The results show clearly that complex formation itself proceeds even at 0 °C although the efficiency of complex formation between preincubated cells and fd at 0 °C was less than half of that at 37 °C. There are at least two possible reasons for the low level of complex formation at 0 °C. One is that the fd adsorption rate at 0 °C is less than half that at 37 °C as with the RNA phage, MS2 (Date, 1979). The other is that the reduction in complex formation is caused by cell aggregation during preincubation at 37 °C (we have often observed such aggregation after preincubation).

Marco *et al.* (1974) reported that M13 binding was completely abolished at 0 °C even though they used cells preincubated at 37 °C for 10 min. The complex formed at 0 °C seems to be fairly loose or fragile and, therefore, may not be detected when complex formation is assayed by centrifugation. Furthermore, 10 min preincubation appears to be insufficient for full activity, as shown in Fig. 1. Tzagoloff & Pratt (1964) reported that M13 attachment could proceed even at 0 °C or in the presence of KCN. The effect of the reaction temperature of 0 °C is apparently similar to our result but we rather favour the explanation given by Marco *et al.* (1974). Tzagoloff & Pratt (1964) used cells that had been starved and then centrifuged. Furthermore, the reaction mixture they used contained only NaCl. Because of starvation, these centrifuged cells may have given little complex formation even at 37 °C and thus apparently positive results even at 0 °C or in the presence of KCN.

We have also shown that complex formation by preincubated cells was lowest on shift-down from 37 to 20 °C. Around this temperature, the cellular ATP concentration was also lowest and both ATP concentrations and complex formation were increased above or below this temperature. Complex formation was also inhibited by the addition of various energy inhibitors or uncouplers to the reaction mixture at 37 °C. Even after a short exposure to these agents at 37 °C (but not at 0 °C) preincubated cells lost the ability to form a complex at 0 °C. The results shown above suggest that when the cell energy level is diminished by exposing cells to a temperature around 25 °C or to energy inhibitors, fd-male cell complex formation is inhibited because of the disappearance of pili from the cell surface. The number of pili per cell was significantly decreased by a temperature shift to 25 °C or by the addition of inhibitors (Table 1), confirming the previous report of Novotny & Fives-Taylor (1974). In the same report these authors showed, however, that M13 adsorption was not affected by exposure of cells to NaCN at 37 °C. This is in contrast to the results shown here. Similarly Novotny & Fives-Taylor (1978) reported that at high temperature (48 or 50 °C but not

46 °C) pili disappeared from the cell surface but this disappearance was not accompanied by a loss of M13 adsorption. Our results (Fig. 4) show that complex formation at 0 °C was abolished by a temperature shift to 50 °C for 3 min. The discrepancy between our results and theirs may be due mainly to the different sensitivity of two assay systems. As we discussed, the adsorption assay by centrifugation which they used appears not to be sufficiently sensitive and also to be affected by mechanical forces.

Marco *et al.* (1974) reported complete abolition of M13 adsorption in the presence of KCN. However f2 binding was still maintained near half the control level. They concluded from their results that binding of M13 to cells requires active metabolism. An alternative interpretation is that pili are not removed so drastically by KCN. We also observed significant adsorption of RNA phage MS2 in the presence of energy inhibitors and uncouplers. However, since RNA phages adsorb to the sides of pili, it is possible that only a few pili remain on the cell surface or in the cell fraction in a free form able to adsorb such phages. From the results described above we conclude that binding of fd phage to pili attached to cells is not likely to be energy-dependent.

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