Role of Membrane Potential and ATP in Complex Formation between *Escherichia coli* Male Cells and Filamentous Phage fd

By MITSUYO YAMAMOTO, SHIRO KANEGASAKI* AND
MASANOSUKE YOSHIKAWA

*Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai Minato-ku,
Tokyo 108, Japan*

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Mutant strains of *Escherichia coli* male cells defective in Ca^{2+} , Mg^{2+} -dependent ATPase (*unc*) were constructed and tested for their ability to form a complex between sex pili and the filamentous phage fd under conditions where either the membrane potential or the cellular concentration of ATP was lowered. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone and the respiratory inhibitor cyanide, as well as valinomycin- K^+ and colicin E1, all markedly diminished complex formation, indicating that the maintenance of a membrane potential, but probably not the pH gradient, is essential for the formation of the complex. Since complex formation with freshly centrifuged cells (which initially lacked sex pili) as well as with preincubated cells (in which pre-existing pili were available for complex formation) was inhibited by exposure to the inhibitors, energy seems to be required for both the reappearance (probably assembly) and the maintenance of sex pili on the cell surface. Brief exposure of freshly centrifuged cells to arsenate resulted in only partial inhibition of complex formation. However, marked inhibition of complex formation was observed following exposure to arsenate of preincubated cells possessing sex pili. This indicates that compounds such as ATP may also be required for maintenance of sex pili on the cell surface.

INTRODUCTION

Extensive genetic analysis (Achtman, 1973; Tomoeda *et al.*, 1975; Rowbury, 1977) has shown that sex pili play an essential role in bacterial conjugation and in infection by specific phages. However, the precise function and the mechanism of assembly and maintenance of sex pili are still not understood. We have previously shown that the formation of a complex between *Escherichia coli* male cells and the filamentous phage fd was greatly reduced by various energy inhibitors, uncouplers and arsenate (Yamamoto *et al.*, 1980). In the same paper, we demonstrated that lowering the cellular ATP concentration by a temperature shift was accompanied by reduced ability to form the complex.

In our previous studies (Yamamoto *et al.*, 1980), the formation of the complex itself was not found to be energy dependent and proceeded even at 0 °C. Since complex formation was prevented by addition of inhibitors to male cells which possessed pili or to cells which had been stripped of sex pili by centrifugation, we concluded that a source of energy was necessary for the reappearance and maintenance of sex pili on the cell surface. Reappearance of sex pili was only partially prevented by chloramphenicol, suggesting that it represented mainly reassembly rather than resynthesis, but energy inhibitors still reduced complex formation in the presence of chloramphenicol, indicating that the assembly was energy dependent. A similar conclusion was also drawn by Novotny *et al.* (1972) and O'Callaghan *et al.* (1973), based on electron microscopic observations of sex pili. Since inhibition of complex formation

by uncouplers or arsenate was not reversed by the addition of glucose or D-lactate, respectively, it was suggested that the energy was not supplied solely by ATP from glycolysis or by electron transport derived from the D-lactate system (Larsen *et al.*, 1974; Yamamoto *et al.*, 1980).

In this report the source of energy for functioning sex pili, which can be assayed by complex formation between male cells and filamentous phage fd, was investigated using *E. coli* male cells defective in the F_0 or F_1 portion of the Ca^{2+} , Mg^{2+} -dependent ATPase (*unc* mutants: Cox *et al.*, 1973). Such bacteria have a lowered membrane potential without an accompanying lowering of the cellular ATP concentration (Plate *et al.*, 1974; Plate, 1979) when treated with uncouplers, valinomycin or colicin E1. Our results suggest that the maintenance of a membrane potential is essential for the formation and maintenance of sex pili.

METHODS

Bacteria and phage. *Escherichia coli* K12 strain YC233 (a *met*⁺ derivative of W1895 Hfr) was used as the parental male strain (Yamamoto *et al.*, 1980). An *uncB* derivative (YM-22) of this strain was isolated after phage P1 transduction with strain YC233-8 (an *ilv* derivative of YC233) as recipient and *E. coli* strain AN382 (*argE3*, *thi*, *str*, *uncB401*) as donor. Strain YC233-8 (*ilv*) was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (50 µg ml⁻¹) treatment of YC233 followed by penicillin screening. Strain AN382 was kindly provided by F. Gibson, Australian National University, Canberra, Australia. *UncA* and *uncB* mutants carrying *Flac* (YM-11 and YM-21) were prepared by transfer of *Flac* to *E. coli* KY2377 (*lac-1*, *gal-1*, *gal-2*, *tonB*, *uncA401*) and *E. coli* OK101 (*lac-1*, *gal-1*, *tonB*, *uncB402*). Strains OK101 and KY2377 were kindly provided by M. Futai, Okayama University, Okayama, Japan. Since we observed frequent reversion of *uncB* mutants, a single colony, which we used as the source of the culture in each experiment, was checked to determine if it grew in M9 medium with succinate as sole carbon source. Filamentous phage fd was labelled with $H_2^{35}SO_4$ as described by Yamamoto *et al.* (1980).

Filtration assay of fd complex formation. This was done as described previously (Yamamoto *et al.*, 1980). ³⁵S-labelled phage fd [5000 to 12000 c.p.m., approximately 5.0×10^4 plaque forming units (c.p.m.)⁻¹ in a fresh preparation] was added to 0.2 ml cell suspension, and the mixture was incubated under the conditions described in the figure legends. The mixture was filtered through a cellulose acetate filter (Gelman Metricel GA6), the filter was washed with 30 mM-Tris/HCl buffer, pH 7.4, containing 0.8% (w/v) NaCl, and radioactivity retained on the filter was counted. In general, complex formation was assayed using centrifuged cells, most of which initially did not possess sex pili. Sex pili appeared during the incubation at 37 °C (Yamamoto *et al.*, 1980). Therefore, addition of reagents to this system should affect the assembly or maintenance of sex pili or both. Preincubation of centrifuged cells at 37 °C resulted in extension from the cells of pili ready to be used for complex formation. Complex formation between fd and pre-existing pili occurred even at 0 °C. In order to determine the effect of reagents on the maintenance (or functional activity) of sex pili, therefore, the reagents were added at the end of preincubation and complex formation was assayed at 0 °C. Complex formation is expressed as the percentage of phage retained on the filter based on the radioactivity added to the reaction mixture as 100%.

Valinomycin treatment. Following the basic methods of Szmecman & Adler (1976) and Plate (1979), bacteria grown in L-broth to a concentration of about 1.6×10^9 cells ml⁻¹ were harvested by centrifugation and washed with 0.1 M-Tris/HCl, pH 7.8. The pellets were resuspended in 1 ml of the same buffer. After keeping the suspension for 6 min at 37 °C, Na₂EDTA (10 mM)/Tris (15 mM), pH 8.0, was added and incubation was continued for a further 3 min; 10 ml ice-cold 0.1 M-sodium phosphate buffer (pH 7.2) was then added to the reaction tubes, and the mixtures were centrifuged. The bacteria were suspended in 3 ml 10 mM-sodium phosphate buffer, pH 7.2, containing 0.1 M-NaCl, and then mixed with the same volume of 10 mM-sodium phosphate buffer, pH 7.2, supplemented with 22 mM-glucose, 20 mM MgSO₄, 40 mM-K⁺ (as potassium phosphate buffer), 0.1 M-NaCl and 10 µM-valinomycin. The reaction mixture without valinomycin was used as a control. The final suspension was incubated with shaking at 27 °C for 4 min and kept in ice until used.

Assay of cellular ATP concentration. ATP concentration was measured by the luciferin-luciferase assay (Lundin & Thore, 1975) as described by Yamamoto *et al.* (1980). Bacterial suspensions (0.5 ml) were mixed with 2 ml boiling buffer (20 mM-Tris/HCl containing 2 mM-EDTA), and the mixture was boiled for 90 s to extract ATP. A portion of boiled solution (0.5 ml) was mixed with 0.2 ml of the firefly extract solution and light emission was recorded using a Bioluminescence reader (BLR-101C; Aloka Co., Tokyo, Japan). Protein was determined by the Lowry method using bovine serum albumin as a standard.

Fig. 1

Fig. 2

Fig. 1. Effect of CCCP and arsenate on complex formation between phage fd and centrifuged cells of strain YM-22 (*uncB* Hfr). A suspension of centrifuged cells (3.5×10^8 in 0.2 ml L-broth, see Methods) was first treated for 3 min with either CCCP (final concentration 50 μ M, ■) or arsenate (final concentration 10 mM, □), or left untreated (○, ●), and then mixed with 35 S-labelled phage fd (15 μ l, 5700 c.p.m.) and incubated at 37 °C (■, □, ○) or 0 °C (●) for the indicated periods. Complex formation was assayed by filtration as described in Methods.

Fig. 2. Complex formation at 0 °C between phage fd and preincubated cells of strain YM-22 (*uncB* Hfr). A suspension of centrifuged cells (3.5×10^8 in 0.2 ml L-broth) was incubated at 37 °C for 27 min and then treated for 3 min with either CCCP (final concentration 50 μ M, □) or arsenate (final concentration 10 mM, Δ), or left untreated (○). After cooling for 3 min in an ice-water bath the suspension was mixed with 35 S-labelled phage fd (10 μ l, 10000 c.p.m.) and incubated at 0 °C for the indicated periods. Complex formation was assayed as described in Methods. For comparison, complex formation of non-preincubated cells at 37 °C (■) and at 0 °C (●) is also shown.

Chemicals. Purified colicin E1 was a gift from A. Nakazawa, Yamaguchi University, Ube, Japan. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma. Firefly lantern extract and ATP were obtained from Calbiochem. All other chemicals used were reagent grade.

RESULTS

Complex formation between male cells defective in Ca^{2+} , Mg^{2+} -dependent ATPase (*unc*) and filamentous phage fd. Hfr bacteria defective in the *uncB* gene, and *uncA* or *uncB* mutants carrying *Flac* were constructed as described in Methods and tested for complex formation with 35 S-labelled phage fd. Slightly less complex was observed with the *unc* derivatives as compared with the wild-type parent.

Effect of CCCP and cyanide on complex formation between *unc* male cells and phage fd. Uncouplers of oxidative phosphorylation are known to deplete the energized membrane state. In a previous paper (Yamamoto *et al.*, 1980), we showed that such uncouplers inhibited complex formation between *E. coli* male cells and phage fd. Using *unc* mutants (*uncB* Hfr, *uncA* *Flac* and *uncB* *Flac* strains), in which the energized membrane state depends solely on electron transport and not on ATP hydrolysis mediated by ATPase (Harold, 1977), we confirmed that the uncoupler CCCP inhibited complex formation at 37 °C (Fig. 1) when added to freshly centrifuged bacteria without preincubation. Similar results were obtained using the respiratory inhibitor NaCN. The results suggest that reappearance of sex pili requires the energized membrane state.

Even brief (3 min) exposure to CCCP at 37 °C at the end of the preincubation of the bacteria diminished subsequent complex formation measured at 0 °C (Fig. 2). Again, similar results were obtained with NaCN. The results indicate that sex pili extended from the cell surface during preincubation (Yamamoto *et al.*, 1980) but were quickly lost upon exposure to

Fig. 3. Complex formation between phage fd and valinomycin-treated cells of (a) strain YC233 (*unc*⁺) and (b) strain YM-22 (*uncB* Hfr). A suspension of cells (8×10^8 in 0.2 ml) which had been treated with valinomycin (see Methods) in the presence (●) or absence (□) of K⁺, or left untreated (○), was mixed with ³⁵S-labelled phage fd (15 μ l, 7600 c.p.m.) and incubated at 37 °C for the indicated periods. Complex formation was assayed as described in Methods.

Fig. 4. Effect of colicin E1 on complex formation between phage fd and centrifuged cells of strain YM-22 (*uncB* Hfr). A suspension of centrifuged cells (3.5×10^8 in 0.2 ml L-broth) was mixed with 40 μ l of either colicin E1 (10 μ g ml⁻¹, purity 80%; ●) or water (○), and then ³⁵S-labelled phage fd (10 μ l, 12000 c.p.m.) was added and the mixture was incubated at 37 °C for the indicated periods. Complex formation was assayed as described in Methods.

the uncoupler or the respiratory inhibitor, i.e. maintenance of sex pili required the energized membrane state.

Effect of valinomycin and colicin E1 on complex formation between unc male cells and phage fd. Valinomycin, in the presence of K⁺, is known to lower the membrane potential without affecting the pH gradient established in the bacteria (Ramos & Kaback, 1977). Colicin E1 also induces membrane depolarization by increasing the permeability to ions (Tokuda & Konisky, 1978, 1979). We therefore tested whether these agents inhibited complex formation at 37 °C between phage fd and *unc* derivatives of an Hfr strain or *unc* mutants which possessed *Flac*.

Fig. 5. Effect of valinomycin on the cellular ATP concentrations of (a) strain YC233 (*unc*⁺) and (b) strain YM-22 (*uncB* Hfr). A suspension (0.5 ml) of valinomycin-treated (●) or untreated (O) cells was incubated at 37 °C for the indicated periods. Cellular ATP concentrations were assayed as described in Methods.

As shown in Fig. 3, addition of valinomycin in the presence of 20 mM-K⁺ to freshly centrifuged, EDTA-treated *uncB* Hfr bacteria significantly reduced complex formation, as observed with the parental strain. Similar results were also obtained with other *unc* derivatives of male strains. Colicin E1 also inhibited complex formation (Fig. 4). The cellular ATP concentrations of an *uncB* Hfr strain and the parental strain treated with valinomycin are shown in Fig. 5. The ATP concentration in *unc* mutants was either only slightly lowered or not affected compared with that in wild-type strains in all cases tested. These results indicate that the maintenance of a membrane potential, but probably not the pH gradient, is essential for the reappearance of sex pili.

Effect of arsenate on complex formation in the unc strains. Arsenate is known to lower cellular ATP concentrations drastically (Klein & Boyer, 1972). If the membrane potential is the only source of energy for functioning sex pili, complex formation should be relatively resistant to arsenate. As shown in Fig. 1, arsenate partially inhibited complex formation between freshly centrifuged *unc* Hfr bacteria and phage *fd*. The effect of arsenate was also studied using preincubated cells in which sex pili were already induced and ready to use for complex formation. Addition of arsenate at the end of preincubation resulted in a marked reduction of complex formation (Fig. 2), suggesting that, in addition to changes in membrane potential, decreased cellular ATP concentrations also influenced the maintenance of sex pili.

DISCUSSION

In our previous paper (Yamamoto *et al.*, 1980), we showed that the ability of *E. coli* male cells to form a complex with phage *fd* depended on the number of sex pili observed on the cell surface and that complex formation itself was not energy dependent and proceeded even at 0 °C. Thus, the extent of complex formation reflects the number of functioning sex pili on the cell surface. In the present study, we have shown that complex formation between *E. coli* male cells deficient in Ca²⁺, Mg²⁺-dependent ATPase (*unc*) and filamentous phage *fd* was inhibited strongly by exposure of freshly centrifuged bacteria to the uncoupler CCCP, the respiratory inhibitor NaCN, valinomycin-K⁺ and colicin E1. In contrast, complex formation was only partially inhibited by arsenate. Little or no diminution in the cellular ATP concentration of the *unc* mutants was produced by exposure to the inhibitors other than arsenate, whereas

drastic lowering was observed in the presence of arsenate (results not shown). In the parental strain, the ATP concentration was lowered to 30 to 50% of normal by uncouplers or by NaCN and to less than 3% by arsenate (Yamamoto *et al.*, 1980). These results indicate that the energized membrane state is a major requirement for the formation of functionally active sex pili capable of forming a complex with phage fd. The maintenance of a membrane potential, but not the pH gradient, appears to be essential since valinomycin- K^+ and colicin E1 were both as effective as CCCP in inhibiting eventual complex formation.

Complex formation between preincubated cells and phage fd was also inhibited by subsequent depletion of the membrane potential or by lowering of cellular concentrations of ATP (and possibly of related so-called 'high-energy compounds'). The preincubated cells acquired sex pili on the cell surface, apparently ready for complex formation. This was in contrast to freshly centrifuged bacteria, which initially did not possess sex pili and failed to form the complex. These results indicate that both the membrane potential and compounds such as ATP are required for keeping functionally active sex pili on the cell surface. However, we cannot define at the moment how the energy is used in maintaining sex pili.

It is unlikely that the prevention of complex formation represents side effects of the reagents used, since they did not alter complex formation at 0 °C, indicating that the compounds had no direct effect on sex pili (Yamamoto *et al.*, 1980).

We conclude that in the earliest step of filamentous phage infection, the host bacteria must maintain an energized membrane state in order to keep sex pili on the bacterial surface. Sex pili seem to extend from the inner membrane, probably at the place where the inner and outer membranes are joined together (Bayer, 1975); this presumably allows phage adsorbed to the tip of pili (Caro & Schnös, 1966) subsequently to reach the cell surface where single-stranded DNA is introduced into the bacteria. In bacterial conjugation, a source of energy appears to be essential in both male and female cells in order to accomplish DNA transfer and, at least in female cells, this was reported to be the proton-motive force (Grinius & Beržinskienė, 1976).

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