Light-microscopic Visualization of F and Type 1 Pili

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Methods for the direct visualization of F and type 1 pili of *Escherichia coli* in the light microscope are described. The method for visualizing F pili is based on the specific adsorption of fluorescent dye-labelled RNA phages to F pili. The best results were obtained with MS2 phages labelled with rhodamine B. Semi-quantitative determination of the amount of F pili is possible. Type 1 pili can be visualized rapidly and specifically by indirect immunofluorescence. Other structures on the cell surface are neither detected by, nor interfere with these assays. By using different fluorescent dyes the two methods can be combined and both F and type 1 pili can be determined in the same sample.

**INTRODUCTION**

F pili, filamentous appendages on the surface of male *Escherichia coli* strains, are determined by the F genes carried by sex factors (Brinton *et al.*, 1964). Studies on the role of F pili have been hampered by the lack of a simple and reliable assay for their presence and structure. All known assays are based on the specific adsorption of F-specific RNA phages to F pili (Crawford & Gesteland, 1964). Electron microscopy (Brinton & Beer, 1967; Lawn, 1967) is suitable for most purposes; F pili with RNA phages attached to them are easily distinguished from other filamentous appendages, especially type 1 pili (Brinton, 1959), which closely resemble F pili. For routine assays, however, electron microscopy is too slow. Filter binding tests of radioactively labelled phages have been used for routine quantitative measurements (Ippen & Valentine, 1965). This method works satisfactorily with F pili attached to bacteria, but is not applicable to isolated single pili as they are not quantitatively retained by the filter (Danziger & Paranchych, 1970). A technique for determining relative amounts of F pili by their serum-blocking power has also been described (Novotny & Lavin, 1971). This method is not suitable as a routine assay as it measures only the number of adsorption sites and gives no information about size, aggregation or biological activity of the pili.

The presence of type 1 pili on the surface of individual bacteria (Curtiss *et al.*, 1969) can be detected directly only by electron microscopy.

In this paper we present simple assays for the presence and structure of F and type 1 pili. They are based on the visualization of F pili using F-specific RNA phages labelled with a fluorescent dye and, for type 1 pili, on indirect immunofluorescence.

**METHODS**

*Materials.* FITC, RITC and fluoresceinamine were from Sigma, and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole, dansyl chloride and fluorescamine were from Serva (Heidelberg, FRG). DTAF was prepared according to Blakeslee & Baines (1976). FITC-conjugated antiserum against rabbit IgG was purchased from Miles Laboratories, Elkhart, Indiana, USA.

*Strains of E. coli.* Strains Hfr Hayes (ATCC 23739), Hfr Cavalli (ATCC 25256) and K12 F lac (ATCC 23725) were obtained from the American Type Culture Collection. Strain K12 1200 F* (rha, sth)* was obtained from Dr H. Hoffmann-Berling (Dürwald & Hoffmann-Berling, 1968).

*Abbreviations:* DMF, dimethylformamide; DTAF, dichlorotriazinylaminofluorescein; FITC, fluoresceiniso-thiocyanate; RITC, rhodamine B-iso-thiocyanate.

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concentration of 10^5 ml l^{-1}. This dilute solution can be stored in the dark for weeks at room temperature. The acid by precipitation with polyethylene glycol, DEAE-cellulose chromatography and Sepharose 4B gel chromatography.

*piliation of bacteria* was assayed by mixing fluorescent phages with a portion of a bacterial culture to a final phage particle concentration of 10^13 ml^{-1}. This dilute solution can be stored in the dark for weeks at room temperature. The F piliation of bacteria was assayed by mixing fluorescent phages with a portion of a bacterial culture to a final phage particle concentration of 10^12 ml^{-1}, corresponding to a ratio of 1 \times 10^9 to 5 \times 10^3 phage particles per cell (approximately 5 \times 10^1 to 2.5 \times 10^2 plaque-forming units of MS2 or 1 \times 10^1 to 5 \times 10^1 plaque-forming units of Qb). After 5 min at room temperature, samples (1 \mu l) were transferred to clean glass slides and observed with a Zeiss model IM 35 microscope, using incident light fluorescence equipment. For photography, samples were fixed by addition of glutaraldehyde to a final concentration of 0.25% (w/v). Isolated pili were diluted with Tris-saline to an antibody type 1 piliation of bacteria could be demonstrated by agglutination tests. For simultaneous observation of F and type 1 pili, RITC-labelled MS2 particles (2 \times 10^{10} particles) were added to the above solution. The fluorescence of F pili faded slowly and had vanished at about 15 min after the chase.

**Preparation of antibodies against type 1 pili.** Rabbits were immunized with purified type 1 pili (see accompanying paper; Biebricher & Düker, 1984) using standard methods. The IgG fraction was purified from the antiserum by standard methods (McKinney & Spillane, 1975). Purified type 1 pili were mixed with anti-type 1 pili IgG in Tris-saline and incubated overnight at 4°C. The precipitate was centrifuged and the supernatant discarded. The residue was extracted with 0.05 M-glycine/HCl buffer (pH 3.5) and the denatured type 1 pili were sedimented at 20000 g. The pH of the supernatant was adjusted to 7.5 with 1 M-Tris base and stored frozen.

**Assay of type 1 pili by indirect immunofluorescence.** The monospecific anti-type 1 pili IgG preparation (first antibody) was diluted with Tris-saline to a protein concentration of 25 \mu g ml^{-1}. The commercial FITC-conjugated anti-rabbit IgG (second antibody) was diluted 20-fold to a protein concentration of 400 \mu g ml^{-1}. Samples (10 \mu l) were incubated with 1 \mu l first antibody solution for 5 min. Second antibody (1 \mu l) was added and 1 \mu l of the mixture was examined under the microscope without further treatment. The final concentrations of antibodies in the assay solution were 2 \mu g ml^{-1} (first antibody) and 30 \mu g ml^{-1} (second antibody). Under these conditions (first antibody limiting) we did not observe interference of the visualization by immunoprecipitates of first with second antibody, or by agglutination of type 1 piliated cells. Washing steps were thus unnecessary. Increasing the amount of first antibody induced aggregation of free type 1 pili and type 1 piliated bacteria. At high concentration of first antibody type 1 piliation of bacteria could be demonstrated by agglutination tests. For simultaneous observation of F and type 1 pili, RITC-labelled MS2 phages (1 \times 10^{10} particles) were added to the above solution.

**SDS-polyacrylamide gel electrophoresis.** This was performed in 17% (w/v) acrylamide gels according to Laemmli (1970).
Visualization of pili

Spectra. Absorbance spectra of labelled phage preparations in visible and UV light were measured on a Cary 118 spectrophotometer. The dye concentration was determined from the specific absorbance of the dye at the corresponding wavelength, assuming equal absorbances for bound and for free dye. The phage particle concentration was calculated from the specific absorbance of the labelled phage preparation at 260 nm, neglecting the small absorbance contribution of the dye molecule at this wavelength. Fluorescence emission and excitation spectra of labelled phage preparations were measured with an Aminco Bowman spectrophotofluorimeter and compared with spectra of unbound dye as reference.

Electron microscopy. Samples were prepared and negatively stained as described by Lawn (1967), and examined with a Siemens electron microscope.

RESULTS AND DISCUSSION

Principle of the method

F pili and other appendages on the surface of bacteria have diameters far below the resolution of the light microscope, even though they may be longer than the bacterial cell. Although the visualization of thicker bacterial flagella by high-intensity dark-field illumination has been reported (Macnab & Koshland, 1973), this has not been possible for F and type 1 pili or F pili covered by F-specific phages. Immunofluorescence microscopical studies (Lazarides & Weber, 1974; Osborn et al., 1978; Weber et al., 1975) have permitted the visualization of submicroscopic intracellular structures in eukaryotic cells, and so we tried a similar approach for the visualization of F and type 1 pili. For type 1 pili we adopted the method of indirect immunofluorescence. Instead of antibodies to F pili, however, we used infectious F-specific RNA phages labelled with fluorescent dye as a specific stain for F pili. If the ability to bind RNA phages is considered as a criterion for the presence of functional F pili (Brinton & Beer, 1967; Crawford & Gesteland, 1964), then the assay is a measure of active pili, since inactive F pili remain invisible. Under optimal conditions labelled phages should appear in the light microscope as tiny light dots, whereas pili covered with RNA phages should be revealed as fluorescent threads. The number of RNA phage particles needed to cover the pilus surface is about 700 per μm of F pilus, as estimated from electron micrographs (Paranchych, 1975). It is critical for the success of the method that the fluorescence intensity at the pilus surface is sufficient for visualization. In order to achieve this, careful selection of the fluorescent dye and of optimal conditions for maximum labelling of the phage with minimal loss of adsorption capacity is required.

Labelling of the RNA phages

For the required specificity of the assay it is necessary to attach the fluorescence label covalently. Fluorescent probes alone (Edelman & McClure, 1968) are not suitable. Vandekerckhove & van Montagu (1974) labelled phage MS2 with fluorescamine. Most of the infectivity of the phage was lost, however, and fluorescamine has the further disadvantage of rapid bleaching. We investigated preparations of the serologically unrelated RNA phages MS2 and Qβ and a variety of fluorescent dyes able to bind covalently with amino residues, including FITC, DTAF (Blakeslee & Baines, 1976), RITC (McKinney & Spillane, 1975), dansyl chloride (Weber, 1952), chloronitrobenzooxadiazol (Ghosh & Whitehouse, 1968) and fluorescamine (Udenfriend et al., 1972). From absorbance spectrum measurements of the labelled phage preparations, approximately 100 to 500 dye molecules were calculated to be covalently attached per phage particle (Table 1). Measurements of the dye content of phage preparations by fluorescence emission gave values in good agreement with those derived from absorbance measurements. The infectivity of the phage preparation was usually reduced by labelling to about half of the original value. This value is acceptable if one takes into account that the conditions for infectivity are much stricter than those for specific binding, and therefore many phage particles unable to give rise to a plaque still adsorb specifically to pili. Of the dyes tested, only RITC, FITC, DTAF and fluorescamine gave preparations of sufficient fluorescence quantum yield to allow visualization in the fluorescence microscope. RITC was found to be outstanding both in brightness of fluorescence and in resistance to bleaching by the excitation light.
Table 1. Properties of labelled phage

<table>
<thead>
<tr>
<th>Phage</th>
<th>Fluorescent dye</th>
<th>Dye molecules bound per phage particle</th>
<th>Infectivity</th>
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<tr>
<td></td>
<td></td>
<td>Specific*</td>
<td>Relative†</td>
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<tr>
<td>MS2</td>
<td>FITC</td>
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<td>13-3</td>
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<tr>
<td>MS2</td>
<td>DTAF</td>
<td>120</td>
<td>5-8</td>
</tr>
<tr>
<td>MS2</td>
<td>RITC</td>
<td>110</td>
<td>4-6</td>
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<tr>
<td>Qβ</td>
<td>DTAF</td>
<td>260</td>
<td>9-3</td>
</tr>
<tr>
<td>Qβ</td>
<td>RITC</td>
<td>350</td>
<td>1-6</td>
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<td>Qβ</td>
<td>Chloronitrooxadiazole</td>
<td>500</td>
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* Percentage of plaque formers among phage particles.
† Infectivity of untreated phage preparation set to 100.
‡ Not determined; fluorophor is formed during reaction.

Fig. 1. SDS gel electrophoresis of labelled phages. $3 \times 10^{12}$ phage particles were denatured for 3 h at 37 °C in a buffer containing 0.5% (w/v) SDS and 0.5% (w/v) 2-mercaptoethanol and loaded on a 17% (w/w) polyacrylamide gel. The gel was deliberately overloaded to emphasize minor components. Lane A, DTAF-Qβ; B, DTAF-MS2; C, RITC-Qβ; D, RITC-MS2; E, fluorescamine-Qβ; F, fluorescamine-MS2; G, MBD-Qβ; H, dansyl-MS2; I, unlabelled Qβ; J, unlabelled MS2. (a) Fixed and stained with Coomassie blue R 250; (b) fluorescence excited by UV light of 254 nm (gel unfixed). Components that reacted with fluorescent dye are numbered: 1, RNA; 2, Qβ coat protein; 3, MS2 coat protein; 4 and 5, unknown low molecular weight constituents of labelled phages. The scale on the left is in centimetres.

As expected, in sucrose gradient centrifugation the fluorescent phages showed the same sedimentation behaviour as unlabelled phages. They banded in CsCl density gradient equilibrium centrifugation as a single, sharp band of precipitated phages. We concluded from these experiments that the physical properties of the phage particles were hardly altered by the labelling procedure except that their solubility was somewhat decreased. SDS gel electrophoresis revealed which component of the phages had reacted with the fluorescent dye (Fig. 1). As expected, the fluorescent dye reacted predominantly with the most abundant component, the coat protein; other proteins in the phage shell, and apparently even the phage RNA, reacted to a minor extent. An additional bright fluorescent band, at a position corresponding to a protein much smaller than the coat protein, was found reproducibly in the phage preparations for most
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Fig. 2. F piliation of F' bacteria. Cells of *E. coli* K12 1200 F' grown in nutrient broth without aeration to late exponential phase were treated with 0.25% glutaraldehyde after addition of labelled MS2, and examined by incident light fluorescence combined with transmitted light phase-contrast. Top, photomicrographs; bottom, drawings to suppress blurring effects of finite depth of field and Brownian motion. (a) Normally piliated cells; (b) highly piliated cells.

of the fluorescent dyes. Since this band moved, in the case of rhodamine labelling, to the anode rather than to the cathode, and since the band was weakly stained with Coomassie blue, it could not have been produced by unreacted fluorescent dye. We did not investigate the source of this band further.

**Conditions for detection of F pili**

The adsorption of F-specific RNA phages to F pili was almost independent of temperature (Brinton & Beer, 1967) and showed no ion specificity, requiring only an adequate ionic strength (0.1 M or greater) for maximum adsorption (Danziger & Paranchych, 1970). Adsorption of labelled phages to F pili took place effectively at room temperature in all bacterial culture media tested and in most buffers in the pH range 5 to 10 (borate-buffered solutions did not work well for some unknown reason). The most critical parameter was the ratio of phage to pili. Low phage concentrations allowed only a partial saturation of the pili with phage, and the image brightness was poor. High phage concentrations, on the other hand, reduced the contrast. At the appropriate ratio range of phage particles to pili, pili appeared as brightly fluorescent flexible rods by incident light fluorescence microscopy (Fig. 2). Fortunately, the upper and lower limits of the suitable ratio range may differ by a factor of at least ten. At a phage particle concentration of $10^{12}$ ml$^{-1}$ (giving a phage:cell ratio of $1 \times 10^3$ to $5 \times 10^3$), the brightness of the fluorescence allowed the simultaneous observation of bacteria and other structures in bright-field or phase-contrast with a dimmed transmitted light source. Attached or free pili were readily discerned. The pili varied in length, from very short stubs up to filaments estimated at 50 μm long. The pili showed Brownian motion and pili attached to bacteria could also be seen to follow the quite often vigorous bacterial and flagellar motion.

The demands on microscope quality were high due to the small size of the pili. Incident light fluorescence equipment with optimal filter combinations and high numeric aperture objectives were required. With the high-quality microscopes we used (see Methods) the visibility of the pili was excellent. Photographic recording was disappointing in comparison to visual observation, and quite often the photographed pili appeared blurred (Fig. 2). This was due partly to the bacterial and Brownian motion mentioned above, and partly to a limited depth of field, which was too small to allow simultaneous focussing on all the pili present on one bacterium.
Spec\textit{ificity of the assay for F pili}

The assay was specific for F pili. F\textsuperscript{−} strains, which do not produce F pili, showed no fluorescent structures. Other filaments present on the bacterial surface neither adsorbed labelled phages nor inhibited their adsorption to F pili. Free pili (Fig. 3) and attached pili had an equal capacity to adsorb labelled phages. The visualization method is therefore useful as a quick and reliable assay for the presence of F pili. Thermal denaturation studies of purified F pili revealed that visualization of F pili by fluorescent phages was not affected by heating the F pili for 10 min up to 70 °C prior to treatment with fluorescent phages; heating to 75 °C, however, rendered F pili unreactive with fluorescent phages, and thus invisible in the assay. This thermal denaturation behaviour is in agreement with published data (Date \textit{et al.}, 1977; Tomoeda \textit{et al.}, 1975) on the effects of heat on adsorption of unlabelled RNA phages to F pili. Apparently the specific binding of most RNA phages is not altered by covalent attachment of dye molecules. Furthermore, there is no non-specific adsorption induced by the presence of dye residues on the phages. In support of this conclusion, fluorescent phages could be chased with an excess of unlabelled phages, causing a slow fading of fluorescence on the pili. Fluorescent phages can thus be displaced by unlabelled phages from the binding sites of the pilus.

Fig. 3. Free purified F pili. (a) Micrograph of F pili visualized with RITC-MS2; (b) electron micrograph of F pili covered with MS2.
Visualization of pili

Does this visualization assay reflect the real appearance of F pili in vivo? The number and activity of F pili as seen in the fluorescence microscope were in agreement with data obtained by electron microscopy (Fig. 3; Lawn, 1967) and filter tests (Beard et al., 1972). Since our method required only the mixing of pili and fluorescent phages, manipulations likely to introduce artefacts were minimized. However, the addition of infectious phages to a sensitive bacterial culture no doubt severely affects the bacteria. Bacteria treated with preparations of MS2 differed in microscopic appearance from those treated with Qβ. Bacteria treated with RITC-labelled MS2 remained single and retained a violent flagellar motion for several hours after phage addition. No lysis or other change of the bacterial shape was detected under these conditions. Free and attached pili were clearly visible and showed little tendency to aggregate. On the other hand, bacteria treated with DTAF-labelled Qβ lost their ability to move actively after a few minutes and tended to aggregate to form larger conglomerates interconnected by intensely fluorescent pili. Free pili showed weaker fluorescence than attached pili. This difference in appearance depended on the phage type, not on the dye used, since the same phage preparations labelled with different dyes gave identical results. It appeared that the assay with fluorescent dye-labelled MS2 correlated well with what we know about the piliation of bacteria, and we therefore concluded that the MS2 assay is a reliable method of showing the pattern of piliation in vivo.

Manipulations to improve photographic recording often produced artefacts. Fixation with glutaraldehyde (0.25%) arrested flagellar motion effectively without affecting the microscopic appearance. Attempts to reduce Brownian motion by embedding the visualized pili in Epon, polyacrylamide or agar interfered severely with the assay and produced aggregates of fluorescent material. Increasing the viscosity of the assay medium (e.g. with 1% polyethylene oxide) improved the photographic recording in some cases; however, it also led to a considerable reduction in the length of the pili and often to their detachment from the bacterial surface. Treatment of bacterial cultures (F+ and F−) with organic solvents or with EDTA at concentrations above 10 mm made the bacteria weakly fluorescent and interfered with the assay for pili when fluorescent phages were added. Furthermore, in the late stationary phase some bacteria showed fluorescence in the assay while no pili were observed (Beard et al., 1972; see also the accompanying paper: Biebricher & Duker, 1984). It is not known whether this fluorescence was due to non-specific adsorption of phages to bacteria with altered cell walls; if so, the radioactive filter assay would lead to misinterpretations, while such artefacts are easily discerned in the visualization assay.

Light microscopic visualization of type 1 pili

Visualization of type 1 pili by indirect immunofluorescence was achieved by mixing the bacterial culture with monospecific type 1 pili antibodies from rabbit followed by fluorescent dye-labelled antibodies against rabbit IgG. Purified type 1 pili (see accompanying paper: Biebricher & Duker, 1984) were strongly antigenic, and specific antibodies could be easily obtained from immunized rabbits. The assay was simple; fixing with glutaraldehyde was possible but not necessary. Washing steps to remove excess antibodies were avoided. Unwanted immunoprecipitates were excluded by choosing conditions (see Methods) where anti-1-pilin IgG was limiting. A large excess of anti-rabbit IgG was also avoided since excess dye reduced contrast.

Type 1 piliation of bacteria was readily observed by indirect immunofluorescence (Fig. 4). The majority of bacteria were non-piliated but type 1 piliated bacteria, which usually bore very many short type 1 pili (Brinton, 1959, 1965), were seen to be enveloped by a shining corona. Type 1 pili are much shorter than F pili; nevertheless, the corona was always structured so that some type 1 pili could be discerned. In cases where bacteria contained only one or two pili these were seen as stubs.

The assay was specific, and in the absence of anti-type 1-pili antibody type 1 pili were not visualized. Furthermore, the degree of type 1 pilation, and the length and the number of pili seen by indirect immunofluorescence agreed with those seen in the electron microscope. Free type 1 pili and aggregates of pili were also visualized by the assay. Antibodies against type 1 pili
induced some aggregation of type 1 piliated cells, and particularly of free type 1 pili (see Methods). However, under the conditions described for the assay, aggregation was not a serious problem and could be eliminated entirely by the use of monovalent antibodies.

Visualization of F pili was not influenced by the presence of type 1-pili antibodies. Thus, it was possible to assay both F piliation and type 1 piliation in the same sample by using different dyes for each assay (Fig. 5). Routinely, FITC-labelled antibodies and RITC-labelled MS2 phages were used for the simultaneous visualization of F and type 1 pili.
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