Fluorescence assays for F-pili and their application

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INTRODUCTION

Conjugative pili are extracellular filaments elaborated by Gram-negative bacteria expressing certain type IV secretion systems. They are required at the earliest stages of conjugal DNA transfer to establish specific and secure cell–cell contacts. Conjugative pili also serve as adsorption organelles for both RNA and DNA bacteriophages. Beyond these facts, the structure, formation and function of these filaments are poorly understood. This paper describes a rapid, quantitative assay for F-pili encoded by the F plasmid type IV secretion system. The assay is based on the specific lateral adsorption of icosahedral RNA bacteriophage R17 by F-pili. Bacteriophage particles conjugated with a fluorescent dye, Alexa 488, and bound to F-pili defined filaments visible by immunofluorescence microscopy. F-pili attached to F+ cells and free F-pili were both visible by this method. For quantification, cell-bound bacteriophage were separated from free bacteriophage particles by sedimentation and released by suspending cell pellets in 0-1 % SDS. Fluorescence in cell-free supernatant fractions was measured by fluorometry. The authors present a characterization of this assay and its application to F-pilius formation by cells carrying mutations in the gene for the F-pilus subunit F-pilin. Each mutation introduced a cysteine, which F-pilin normally lacks, at a different position in its primary structure. Cysteine residues in the N-terminal domain I abolished filament formation as measured by fluorescent R17 binding. This was confirmed by measurements of DNA donor activity and filamentous DNA bacteriophage infection. With one exception (G53C), cysteines elsewhere in the F-pilin primary structure did not abolish filament formation, although some mutations differentially affected F-pilus functions.
although unacylated subunits still assemble and function (Grossman & Silverman, 1989; Grossman et al., 1990; Moore et al., 1993; Maneewannakul et al., 1995). Unlike T- and RP4-pilins (Eisenbrandt et al., 1999), F-pilin is not circular.

Ippen-Ihler and colleagues showed that formation of membrane F-pilin requires only the traA and traQ genes (Moore et al., 1981b; Maneewannakul et al., 1993) and provided evidence that membrane F-pilin is the precursor to filament F-pilin (Sowa et al., 1983). Our studies showed that TraQ interacts directly with the C-terminal domain IV of F-pilin (Paiva et al., 1992; Harris et al., 1999), and we proposed that TraQ acts catalytically to escort TraA into the inner membrane.

Once formed, membrane F-pilin is stable as such in cells unable to elaborate F-pili (Sowa et al., 1983). F-pilus assembly from membrane F-pilin substrate requires numerous additional Tra proteins that act at or in association with the cell envelope (Firth et al., 1996; Harris et al., 2001; Harris & Silverman, 2004). Altogether, about half the F DNA assembly from membrane F-pilin substrate requires numerous Tra proteins that act at or in association with the cell envelope (Firth et al., 1996; Harris et al., 2001; Harris & Silverman, 2004). Altogether, about half the F DNA transfer (tra) genes essential for DNA transfer are required for F-pilus assembly and function (Grossman & Silverman, 1989; Firth et al., 1996). These additional proteins form an envelope-associated secretion machine (R. Harris and others, unpublished data), as is also true of other type IV systems (Thorstenson et al., 1993; Grahn et al., 2000; Gilmour et al., 2001; Kumar et al., 2000; Krall et al., 2002).

Several indirect tests exist for the presence or absence of functional F-pili, including conjugal DNA donor activity and sensitivity to bacteriophage that use F-pili as adsorption sites. Direct assays for F-pili have included electron microscopy (Valentine et al., 1969; Curtiss et al., 1969), binding of RNA bacteriophage labelled with $^{32}$P (Valentine et al., 1969), and competitive ELISA (Frost et al., 1985). The last two assays are now rarely used, electron microscopy, though poorly suited to kinetic or other studies requiring high throughput, having by default become the assay of choice for F-pili. Here we describe alternative assays that employ fluorescent bacteriophage that bind specifically to F-pili. We show that fluorescence microscopy can be used to analyse F-pili number and length distributions within and between populations, whereas fluorescence measurements can be used for rapid, quantitative assays of cell cultures. We illustrate these advantages using a set of F-pilin missense mutants.

### METHODS

#### Strains, plasmids, bacteriophage, and culture conditions.

All strains were derivatives of *Escherichia coli* K-12. JC3272 and JC3272/Flac (JCFL0) have been described (Achtman et al., 1972). HfrH was originally obtained from the Coli Genetic Stock Center at Yale University. Strain AE2386 was derived from MC4100 (Casadaban, 1976). To abolish formation of type I pili, the *finD*:lac gene of strain VL386 (Freitag & Eisenstein, 1983) was introduced into MC4100 by P1 transduction, selecting for a Lac- phenotype. This strain switched to a Lac+ phenotype at high frequency and quickly became stably Lac-. A spontaneous NalR mutant was designated AE2386, Flac (JCFL0; Achtman et al., 1972) was introduced into AE2386 by conjugation with JC3272/F’lac, selecting for NalR Lac+ transconjugants. XK1200/pOX38traA::cat (Anthony et al., 1994) was obtained from Dr Laura Frost, University of Alberta, Edmonton, Canada. TOP10 [relevant markers: *araD139 Δ araE-pilA-]/cat was obtained from Invitrogen and used for tightly regulated traA expression (Guzman et al., 1995). pOX38traA::cat was introduced into TOP10 by conjugation with XK1200/pOX38traA::cat containing pWP901, a traA+ plasmid based on the pUC19 vector backbone (W. Paiva & P. Silverman, unpublished data). AE2248, used as a recipient in mating experiments, is a thr-34::Tn10 derivative of JC3272.

Bacteriophage R17 and the tet transducing bacteriophage F1fus2 (Parmley & Smith, 1988) were from our laboratory stocks. R17 titres were measured by standard agar overlay with HfrH or JC3272/JCFL0 as host. F1fus2 titres were measured as transductant-forming units (t.f.u.) (tetracycline-resistance) with strain K91 as host (Parmley & Smith, 1988).

Where indicated, plasmid pMR119. encoding DsRed-Express (Otto et al., 2004), was introduced by transformation.

Bacteria were grown routinely in Luria–Bertani (LB) medium supplemented with antibiotics as necessary. For bacteriophage binding, the medium was also supplemented with 10 mM CaCl$_2$. Incubation was at 37 °C with vigorous aeration. Growth was monitored by optical density at 600 nm. When used, nutrient broth contained, per litre: 10 g tryptone, 1 g yeast extract, 8 g NaCl and 0.2-2 % (w/v) glucose.

#### Preparation of fluorescent R17.

R17 was prepared from crude lysates by liquid polymer phase partition and isopycnic banding in step CsCl gradients (Yamamoto et al., 1972). After dialysis against P buffer [50 mM TrisHCl (pH 7-6)/0-1 M NaCl/5 mM MgCl$_2$/0-1 mM EDTA], the suspension was made 50 % (w/v) glycerol and stored at −20 °C. The final titre of the preparation used in these studies was 2.5 × 10$^{12}$ p.f.u. ml$^{-1}$ and has been stable for several years.

For conjugation with fluorescent dyes, a portion of the R17 suspension (12–15 ml) was dialysed at 4 °C for 48 h against 2 litres of a solution containing 0-1 M NaHCO$_3$ (pH 8.5)/5 mM MgCl$_2$. The dialysis solution was changed once after 24 h. Alexa 488 carboxylic acid (succinimidy1 ester; Molecular Probes) (1 mg) was dissolved in 0-1 ml anhydrous DMSO and added in 10 µl aliquots to the R17 suspension with gentle stirring and at ambient temperature over a 20–30 min period. After an additional 60 min with gentle stirring, the R17 suspension was loaded onto two linear CsCl gradients (30 ml; $\rho = 1.66-1.226$ g ml$^{-1}$) in P buffer. Centrifugation in the Beckman SW28 rotor was at 25 000 r.p.m. for 18 h at 5 °C. The fluorescent band visible under UV illumination ($\rho = 1.45$ g ml$^{-1}$) was collected, dialysed against P buffer, diluted with an equal volume of 80 % (w/v) glycerol, and stored at −20 °C. Titres were generally about 5 × 10$^{11}$ p.f.u. ml$^{-1}$ and Alexa 488-labeling corresponded to ~10$^{4}$ fluorescent intensity units (FIU) ml$^{-1}$. In earlier experiments, glycerol was removed by dialysis before the preparation was used, but this proved to be unnecessary.

#### R17 binding and fluorescence measurements.

Cells (0.5–1 ml) and R17 (15–40 µl) were mixed at 4 °C and incubated at that temperature for 10 min. Formaldehyde (50 µl of a 16 % solution) was added and the samples incubated for 10 min at ambient temperature. Cells and bound bacteriophage were harvested by sedimentation for 4 min in a microcentrifuge at 10 000 g. Supernatant fractions were carefully removed by aspiration and discarded. Cell pellets were suspended in 1 ml 0-1 % (w/v) SDS. Cells were sedimented, suspended in 1 ml 0-1 % SDS, and sedimentsed a last time. Supernatant fractions were combined.
Fluorescence was measured with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. A blank value consisting of the fluorescence from 0·1% SDS (40 FIU) or, depending on the experiment, from F− cells in 0·1% SDS (50–70 FIU) was subtracted from all readings.

**Fluorescence microscopy.** Cells were grown to an OD600 of 0·3–0·4 in Medium E salts (Vogel & Bonner, 1956) containing 1% Casamino acids/1% tryptophan/1% glucose. Cultures were diluted to an OD600 of 0·1 with PBS and 50 µl applied to a microscope dish (35 mm × 12 mm × 0·17 mm; World Precision Instruments). Before use, dishes were incubated for 10 min in normal goat serum diluted 100-fold in PBS and then rinsed three times with PBS. After 10 min incubation to allow cells to adhere, excess liquid was carefully removed and replaced with 50 µl R17 diluted fivefold in PBS. After 10 min, liquid was removed and an agar disc (2% in PBS, 0·16–0·19 mm thick) immediately placed over the cells. Fixation was with 2% formaldehyde in PBS (10 µl added to the surface of the agarose disc). After 2 min, excess liquid was removed and the cells were examined with a Zeiss LSM 510META laser scanning confocal microscope.

**DNA donor activity and F1 fus2 sensitivity.** Cultures of TOP10/pOX38traA::cat containing traA genes cloned into a pBAD vector (Gazman et al., 1995) (see below) were grown to an OD600 of 0·4. Where appropriate, traA expression was induced by addition of L-arabinose (final concentration 0·2%, w/v) 2·5–3·5 h before the cells were used. For donor activity, 0·5 ml of each culture was subjected to centrifugation. Supernatant fractions were aspirated and the cells suspended in 0·5 ml of a recipient cell culture (AE2248 grown to OD600 0·23). After incubation for 60 min at 37 °C, the cell samples were diluted and 10 µl aliquots plated on media selective for trans-conjugants (chloramphenicol + tetracycline) and for donor cells (ampicillin + chloramphenicol).

For F1 fus2 infectivity, we measured transduction of the bacteriophage tet gene. A portion (90 µl) of the cultures used to measure donor activity was mixed with 10 µl of an F1 fus2 preparation containing 106 t.f.u. Samples were incubated at 4 °C for 15 min, 37 °C for 15 min, and subjected to centrifugation at 10 000 g for 2 min. Cell pellets were suspended in nutrient broth and incubated for 45 min at 37 °C. Portions (10 µl) of serial dilutions were then spotted on LB/tetracycline plates.

**Construction of F-pilin cysteine mutants by site-directed mutagenesis.** With the exception of G64C, which was obtained by mutagenic PCR, cysteine mutations were introduced into traA of plasmid pWP901 using the USE Mutagenesis kit (Amersham Pharmacia Biotech). The primers used for the different mutations are listed in Table 1. We also found it necessary to introduce a third, wild-type primer for all mutations except A33C (Table 1). The reaction product was used to transform XK1200/pOX38traA::cat, selecting for CamR AmpR transformants. Plasmid DNA was isolated and traA inserts sequenced in both directions. Following Manchak et al. (2002), we transferred the mutant and wild-type traA genes into the vector pBAD/Myc-His A (Invitrogen). These plasmids were introduced by transformation into TOP10/pOX38traA::cat.

## RESULTS

### Binding of fluorescent R17 bacteriophage to F-pili

Bacteriophage R17 was conjugated with the fluorescent dye Alexa 488 and purified as described in Methods. By denaturing gel electrophoresis, the R17 coat protein (~14 kDa; Weber & Konigsberg, 1975) contained essentially all of the fluorescence (Fig. 1). In addition to the coat protein, the preparation contained three other proteins visualized after Coomassie blue staining. These corresponded to ~28 kDa, ~37 kDa and ~48 kDa (Fig. 1). Only the 37 kDa protein, which corresponds in size to that of the bacteriophage A or maturation protein (Weber & Konigsberg, 1975), contained traces of fluorescence.

Biebricher & Duker (1984) first showed that fluorescent RNA bacteriophage bound to F-pili could be visualized by fluorescence microscopy. The result of a similar experiment but carried out with current imaging and image processing technology is shown in Fig. 2. The cells themselves, labelled internally by expression of the fluorescent protein DsRed-Express, are clearly visible. F-pili decorated with R17 showed up as fluorescent filaments in cultures of tra+ F' and Hfr strains but not of F− strains (not shown) or strains containing a tra mutation known to abolish F-pilus formation (Fig. 2).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
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<tbody>
<tr>
<td>S4C</td>
<td>CATCGGTCCTGACACAGCTGCGG</td>
</tr>
<tr>
<td>G5C</td>
<td>CCATCGAGCTCCTGACACAGCTGCGG</td>
</tr>
<tr>
<td>S11C</td>
<td>CGGTGCGTGTACGCATGCCATCAGG</td>
</tr>
<tr>
<td>S25C</td>
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</tr>
<tr>
<td>T45C</td>
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</tr>
<tr>
<td>G53C</td>
<td>GATGGTCGGAAAACAGGCGAAGAATTG</td>
</tr>
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<td>G69C</td>
<td>CCGTCGAGGCAAAACAGCGGC</td>
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<tr>
<td>Common primer†</td>
<td>CGACGAGAACCTTCAGGCCAGAAACC</td>
</tr>
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</table>

* Cysteine codons (reverse complement) are underlined.
† Wild-type sequence corresponding to the A33C mutagenic primer.

**Fig. 1.** Gel electrophoresis of proteins from fluorescent R17. An aliquot (15 µl) of R17 conjugated with Alexa 488 as described in Methods was denatured and the proteins separated by SDS gel electrophoresis. Lane 1, Coomassie blue stain; lane 2, illuminated with UV light; lane M, size markers.

**Table 1.** Primers used to generate F-pilin cysteine mutants
Quantification of R17 binding to F-pili

Fluorescence microscopy can quickly provide information about F-pilus number per cell and length distributions, but for some applications a quantitative, sensitive and rapid assay for total cell-attached F-pili would also be useful. We describe such an assay here.

Fluorescent R17 (2000–3000 FIU) was incubated with F or isogenic F cells (0-4–0-7 OD600 units) to allow bacteriophage adsorption. Cells and bound R17 were collected by sedimentation. The pellets were suspended in 0-1 % SDS, which dissolves F-pili but leaves cells intact. Cells were removed by sedimentation and washed once with SDS. Fluorescence of the combined supernatant fractions was then measured. In preliminary assays, fluorescence in supernatant fractions from F or Hfr cells corresponded to 500–800 FIU per OD600 unit, depending on the strain. Fluorescence in equivalent fractions from F cells was 50–70 FIU per OD600 unit. Fluorescence increased linearly with increasing amounts of F+ cells except at very low levels (< 0-1 OD600 unit) (Fig. 3) and it was proportional to culture density during exponential growth (Fig. 4). As the culture left exponential growth (OD600 > 1-5), the ratio FIU/OD600 unit began to diminish (Fig. 4); a stationary-phase culture of the F' lac strain used in this experiment (OD600 3-2) measured 450 FIU per OD600 unit. This reduced level of F-piliation undoubtedly reflects the well-established F- phenocopy effect.

Induction of traA assayed by R17 binding

Rapidity and sensitivity, the two main advantages of the fluorescence assay described above, are especially useful for kinetic experiments. An example is the formation of F-pili after traA induction. We first constructed a plasmid in which the traA gene was expressed from the araBAD promoter (see Methods). This construct was introduced into TOP10/pOX38traA::cat cells. A culture was then induced by addition of arabinose (0-2 %), and R17 binding was followed as functions of time and culture density. An uninduced culture served as a control. R17 binding could be

Fig. 2. Identification of F-pili by immunofluorescence microscopy of bound R17. Cells expressing DsRed-Express were cultured and prepared for microscopy as described in Methods. (a) Strain HfrH. (Substantially the same results were obtained with JC3272/F' lac.) (b) Strain JC3272/F' lac traH80. The traH80 mutation abolishes the formation of F-pili, as do mutations in any of several other tra genes (Firth et al., 1996). Examination of these other tra mutant strains yielded the same results as the traH mutant. Scale bar, 10 μm.

Fig. 3. Quantitative R17 binding as a function of the F+/F- cell ratio. F- strain AE2386 and F+ strain AE2386/F' lac were grown to OD600 values of 0-86 and 0-70, respectively. Mixtures contained the amounts of the F+ strain indicated and amounts of the F- strain to yield a total of 0-4 OD600 units per sample. R17 binding was then measured as described in Methods. This experiment was repeated three times and reproducibly showed non-linearity below about 0-1 OD600 units of F+ cells.
observed in the induced culture within 60 min of arabinose addition and reached a maximum specific activity (FIU per OD600 unit) after 3–4 h (Fig. 5a). In contrast, no binding above background was observed with uninduced cells (Fig. 5a). Note also that traA induction in these conditions drastically curtailed growth (Fig. 5b), an effect probably related to F-pilin overexpression.

Cysteine-containing F-pilin mutants
Similar to studies by Manchak et al. (2002), we have been constructing and characterizing F-pilin missense mutants, in conjunction with structural studies of F-pili necessary to interpret their effects. Here, we use a subset of such mutants to illustrate the utility and one limitation of the methods described above.

F-pilin, which is only 70 amino acids in length, normally lacks cysteines (Frost et al., 1984). We constructed several mutant traA genes, each with a cysteine codon at a different site (Table 2). Four of the mutations were in domain I, one in domain II, one in domain III and three in domain IV (Table 2) (Paiva et al., 1992). (Note that the traA S25C gene also contained a second mutation, A55V.) These mutant traA genes were cloned into a pBAD vector as described in Methods.

All of the mutants accumulated high levels of membrane F-pilin (Fig. 6). Accumulation was dependent on arabinose induction (Fig. 6, lanes 1 and 2). The S4C and G5C mutants appear to accumulate less than the others (Fig. 6, lanes 3 and 4), but we attribute this to the fact that these mutations are within the wild-type sequence used to raise the anti-peptide antibody used in these experiments (Paiva et al., 1992) and hence have altered epitopes.

Even though these mutant F-pilins accumulated to normal or nearly normal levels, not all formed F-pili. Of the nine mutants tested, only three were positive by R17 binding and only one mutant, containing the G64C mutation, supported as much R17 binding as the wild-type control (Table 2). Tests based on conjugal DNA donor activity and filamentous bacteriophage F1 fus2 sensitivity largely confirmed these results (Table 2). Two mutations, A33C and T45C, led to significantly reduced R17 binding; fluorescence microscopy showed that both these mutations, but especially T45C, led to shorter-than-normal filaments (data not shown). Short filaments would not be expected to affect functions, such as Ff phage binding, that require only an F-pilus ‘tip’, and both the A33C and T45C mutants remained sensitive to F1 fus2 and active as DNA donors (Table 2). One mutation, G69C, abolished R17 binding without affecting DNA donor activity or F1 fus2 sensitivity (Table 2). This mutation appears to have specifically altered R17 adsorption sites along the F-pilus side.

DISCUSSION
Notwithstanding the essential role of conjugative pili in horizontal DNA transfer mediated by type IV secretion systems, their structure, function and assembly are not well understood. The work we describe here was undertaken as part of a broader effort to determine how F-pili are formed and how they function. We were interested in developing simple assays that would yield a statistical profile of F-pili in bacterial populations (F-pilus number per cell and length distributions) and allow for comparative studies of total...
Table 2. Structure and functions of F-pilin cysteine mutants

Functional measurements were as described in Methods.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sequence*</th>
<th>Domain I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>R17 binding (FIU per OD600 unit)</th>
<th>DNA donor activity, Ffus2 sensitivity</th>
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*Domain designations I–IV were as described by Paiva et al. (1992).

F-pilus levels in different cell populations or in the same population at different times or after different physiological perturbations. The main advantage of the fluorescence assays we describe here over previous assays (Valentine et al., 1969; Curtiss et al., 1969; Frost et al., 1985) is that all the information can be obtained with only a single reagent. Fluorescent R17 is straightforward and inexpensive to prepare and stable for years, unlike bacteriophage containing [32P]RNA used in filter binding assays. Sample preparation for fluorescence microscopy is certainly no more time consuming or complicated than that for negative staining and electron microscopy, and a single field can yield data on scores of cells.

As with other assays for F-pili, the ones we describe here rely on the lateral binding of RNA bacteriophage to F-pil. This can be misleading. As we have shown here for the G69C F-pilin mutant, and as Grossman & Silverman (1989) and Manchak et al. (2002) have also shown, altered F-pilins can be incorporated into filaments that fail to bind RNA bacteriophage. Such ambiguities might be minimized by using cysteine-reactive fluorescent dyes, rather than fluorescent bacteriophage, in conjunction with cysteine-containing F-pili.

Manchak et al. (2002), examining the effects of single missense mutations of F-pilin, found that in general DNA donor activity and sensitivity to filamentous DNA bacteriophage tracked together. In contrast, several mutations abolished RNA bacteriophage sensitivity with less of an effect on the other two functions. The G69C mutation we describe here evidently belongs in this class. The effects of the G69C mutation can not be attributed to the presence of cysteine at this locus since the G69D mutation had much the same effects (Frost & Paranchych, 1988). Interestingly, neither we nor Manchak et al. (2002), nor the more limited study by Frost & Paranchych (1988), identified F-pilin missense mutants that significantly reduced Ff bacteriophage sensitivity and DNA donor activity without also reducing or abolishing RNA bacteriophage sensitivity. One explanation for these data is that donor activity and DNA bacteriophage sensitivity are relatively robust functions with respect to modest alterations to F-pilin structure, whereas RNA bacteriophage infection is more sensitive.

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