Characterization of Pili Determined by Drug Resistance Plasmids R711b and R778b

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The bacterial drug resistance plasmids R711b and R778b, at present classified in the X incompatibility group, determine pili (designated 711) that resemble F pili morphologically. Like F pili, 711 pili adsorb F-specific filamentous bacteriophages to their tips, though more often in pairs, than singly. However, F-specific RNA-containing bacteriophages are not adsorbed to their sides, and strains carrying the plasmids are resistant to these phages. Pili determined by the only IncFV plasmid F,lac are similar to 711 pili in their phage adsorption properties, but they are serologically different, as are F pili. It is concluded that F, F,lac and 711 pili have basic differences in spite of a morphological resemblance.

INTRODUCTION

This paper describes the results of a search for conjugative pili determined by the bacterial drug resistance plasmids originally assigned to the X incompatibility group (IncX): R6K (Kontomichalou, Mitani & Clowes, 1970), R485 (Hedges et al., 1973), R711b (Hedges, 1974) and R778b (Hedges, 1974). Whilst R711b and R778b both determined filaments with a morphological resemblance to F pili, no pili could be found associated with R6K and R485. Furthermore, strains carrying R711b and R778b were sensitive to the F-specific filamentous bacteriophages, but were resistant to the RNA-containing phages. The only other plasmids determining these phage sensitivity patterns are laboratory mutants of the IncFI plasmid F (Silverman et al., 1968; Ohtsubo, Nishimura & Hirota, 1970; Achtman, Willetts & Clark, 1971) and the IncFV plasmid F,lac (Falkow & Baron, 1962; Datta, 1975). The latter is not to be confused with Flac which is derived from F. F,lac determines pili morphologically similar to those of F (Brinton, 1965; Lawn et al., 1967) and is unrelated by incompatibility to R711b and R778b (N. Datta, personal communication).

This work characterizes the pili determined by R711b and R778b and shows that they differ in some respects from both F and F,lac pili. A detailed study of any one aspect was not intended; the objective was to indicate areas of general interest. F,lac pili are only included in a few of the experiments described since a more detailed description of them (encoded by a derepressed F,lac plasmid constructed by N. Willetts) is being carried out elsewhere (W. Paranchych & N. Willetts, personal communication).

There are now some aspects concerning the classification of R711b and R778b (resistance determined to Km and Su respectively). Both were described as belonging to the incompatibility group X (IncX): R778b was incompatible with the multicopy plasmid R6K (Kontomichalou et al., 1970; designated the prototype IncX plasmid by Hedges et al., 1973), and R711b was incompatible with R778b (Hedges, 1974). Subsequently it has been shown that R711b and R6K are not incompatible; therefore these three plasmids do not all belong to one group on the basis of incompatibility (Datta, 1975; N. Datta, personal communication). In view of this, the new pili have been temporarily designated 711 pili.
Table 1. Bacterial strains used

<table>
<thead>
<tr>
<th>R+ strains</th>
<th>Compatibility group</th>
<th>Description*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> K12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>153-1(R6K)</td>
<td>X†</td>
<td>Ap⁺, Sm⁻, Pil⁺</td>
<td>Datta (Kontomichalou et al., 1970)</td>
</tr>
<tr>
<td>153-2(R711b)</td>
<td>X†</td>
<td>Nal⁺, Su⁺, Pil⁺</td>
<td>Datta (Hedges et al., 1973)</td>
</tr>
<tr>
<td>153-1(R778b)</td>
<td>X†</td>
<td>Rif⁺, Km⁻, Pil⁺</td>
<td>Datta (Hedges, 1974)</td>
</tr>
<tr>
<td>c600(F₆lac)</td>
<td>FV</td>
<td>Lac⁺, Pil⁻</td>
<td>Datta (Falkow &amp; Baron, 1962)</td>
</tr>
<tr>
<td>CR34(F+)</td>
<td>FI</td>
<td>F⁺, Pil⁻</td>
<td>Author</td>
</tr>
</tbody>
</table>

| R- strains       |                     |              |                                |
| *S. typhimurium* LT2 |                 |              |                                |
| SQ1139(sm-r)     | Sm⁻, Pil⁻          | R. Bradley   |                                |
| SQ1139(rif-r)    | Rif⁻, Pil⁻         | Author       |                                |
| SQ1139 D3        | Pil⁻, phage⁻       | Author, see text |                                |

* Abbreviations for drug resistance markers as in Novick et al. (1976). Pil⁺, type I pili present; Pil⁻, type I pili absent.
† Present classification of some X plasmids is incorrect, see text.

METHODS

Bacterial strains and growth media. The strains used are listed in Table 1. Difco Nutrient Broth was generally used as the nutrient medium, 2 % (w/v) agar being added for plates. The exceptions were for strain c600-(F₆lac) which grew better on plates of BBL Brain Heart Infusion Broth with 2 % (w/v) agar, and for plates containing sulphonamide which required BBL Mueller Hinton Agar. Cultures were incubated at 37 °C.

Bacteriophages. The F-specific RNA-containing phages were represented by ZIK/I (Bradley, 1964) and R17 (Paranchych & Graham, 1962). The F-specific filamentous phage used was fd (Marvin & Hoffmann-Berling, 1963), and other filamentous phages were IKe (Khatoon, Iyer & Iyer, 1972) and Pf3 (Stanisich, 1974). The lipid-containing phage PR4 (Stanisich, 1974) was also used.

The filamentous phage f711 was isolated from sewage (St John's, Newfoundland) as follows. Raw sewage (5 ml) was added to a freshly inoculated culture of *Salmonella typhimurium* SQ1139 D3(R711b) containing 20 μg kanamycin ml⁻¹. After overnight incubation (static), bacteria were removed by centrifugation (7000 g, 15 min) and 10 ml of the culture fluids was added to 100 ml of another culture of the same strain. After overnight shaking at 37 °C, cell-free culture fluids were found by electron microscopy to contain many filaments of equal length. Single plaques were obtained on *E. coli* K12-2(R711b) and the phage was purified by standard methods. The filamentous nature of the infectious agent was verified by examining an extract from a single plaque in the electron microscope.

All bacteriophage suspensions were in broth and were prepared by extraction of confluently lysed soft agar plates of host bacterium, or by infection of liquid cultures followed by 24 h incubation with shaking for filamentous phages.

Matings. Plasmids were transferred from *E. coli* 153-2(R711b) and 153-1(R778b) to *S. typhimurium* SQ1139 (see Table 1) using standard mating procedures. Nutrient broth shake cultures were inoculated from fresh overnight streaks of R⁺ donors grown on selective plates (see below). Cultures were grown to the early-exponential phase, then adjusted to an extinction of 0.2 at 620 nm. Since the crosses were intergeneric, the recipients were incubated for 30 min at 50 °C just before mating to increase the yield of transconjugants by reducing restriction (Mojica-a & Middleton, 1971). Donors and recipients were mixed in equal volumes to give a ratio of about 1:1 and incubated statically for 1.5 h at 37 °C. Portions (0.1 ml) of serial dilutions of the mating mixture in broth were spread on selective plates which were incubated overnight, or until transconjugants grew. Rifampicin (100 μg ml⁻¹) or streptomycin (200 μg ml⁻¹) were used for counter selection against donors. Drug concentrations for plasmid selection were (μg ml⁻¹): R711b, kanamycin, 20; R778b, sulphonamide, 1000.

Titre increase test. This was used to complement the standard spot test for sensitivity to bacteriophages. The method used applies only to filamentous phages which are produced without lysing their bacterial hosts.

A 4 h shake culture of the strain under test (extinction 0.15 at 620 nm) was inoculated with a suspension of fd to a final concentration of 1 x 10⁶ plaque-forming units (p.f.u.) ml⁻¹ and incubated with shaking for 24 h to allow maximum phage production (phages other than filamentous types are usually incubated for a few hours only). Bacteria were then removed by centrifugation, and the supernatant was titrated in triplicate for...
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free phage. A blank, consisting of broth containing fd at $1 \times 10^4$ p.f.u. ml$^{-1}$, was treated concurrently. The increase in titre was calculated as a ratio by dividing the value obtained from the bacterial culture by that from the blank.

Efficiency of phage adsorption by bacterial strains. This was determined by mixing 5 ml of an exponential phase culture containing $1 \times 10^8$ cells ml$^{-1}$ with an equal volume of fd at $1 \times 10^4$ p.f.u. ml$^{-1}$, incubating for 20 min and then centrifuging to remove bacteria. The culture fluids were titrated in triplicate for unadsorbed phage. The percentage of fd adsorbed was calculated by subtracting the titre from that of a blank (no bacteria) which represented 0% adsorbed phage.

Electron microscopy. For pilus counts, bacterial cultures which had been inoculated from overnight selective plates were shaken at 37°C for 5 h, then incubated statically for a further 2 h; the latter stage gave optimum conditions for pilus production by allowing pili to grow without breakage due to shaking. After gentle agitation to resuspend any sedimented cells, a carbon-coated electron microscope specimen support grid was touched on the surface. Excess liquid was removed with a filter paper and the grid was allowed to dry at the extreme edge only. It was then washed twice by floating on 0.1 M-ammonium acetate solution, and negatively stained with 0.15% (w/v) sodium phosphotungstate solution. The number of pili cell was estimated by counting pili on 50 cells in the electron microscope.

The lengths of pili were measured directly from electron micrographs using a ruler for straight pili and a map measurer for bent ones (calibration: polystyrene latex). The values given in Results are for protruding lengths; correction for obscuration by the cell (Novotny, Carnahan & Brinton, 1969) was not considered necessary since the measurements were for comparative purposes only. Cultures used were the same as for pilus counts, individual cells being photographed at random.

Preparation of antisera and immune electron microscopy. Rabbits were inoculated intravenously approximately every other day for 3 weeks with a pilus suspension (0.1 ml increasing to 1.0 ml for the last five injections) prepared as follows. A bacterial strain carrying the appropriate plasmid was streaked on the entire surface of six nutrient plates containing a selective antibiotic. (Plate cultures gave far more pili than broth cultures, but the numbers were too inconsistent for counting purposes.) After overnight incubation, cells were scraped off and resuspended in about 2 ml of phosphate-buffered saline. This suspension was placed in a Thomas Teflon Pestle Tissue Grinder (volume 55 ml, clearance 0.15 to 0.23 mm, serrated pestle), operated at 200 to 500 rev. min$^{-1}$ for about 2 min. Cells were removed by centrifugation and the suspension was checked for pilus content by electron microscopy. This method sheared 711 pili from the cells very well giving many filaments with little broken cell debris in the final suspension. Type 1 pili were present in very small numbers. The rabbits were bled 2 weeks after the last injection, and the serum obtained was heated to 56°C for 40 min to remove complement.

Two techniques were used for labelling pili with antibodies. In the first (Lawn, 1967), bacteria were suspended in a drop of broth from a fresh plate culture on selective medium to a high concentration ($> 1 \times 10^8$ cells ml$^{-1}$) and a carbon-coated electron microscope specimen grid was touched on the surface. The grid was washed by floating on 0.1 M-ammonium acetate solution for 30 s. It was transferred to a mixture of 1 vol. antiserum and 4 vols 0.1 M-ammonium acetate solution for 5 min at room temperature, then washed three times (at least 1 min each) in separate baths of ammonium acetate, and negatively stained in 1% (w/v) sodium phosphotungstate. The second, more sensitive procedure was derived from that of Lawn & Meynell (1970). Bacteria from a plate culture were suspended in a 1:1 or 1:20 dilution of homologous antiserum in ammonium acetate (1:5 for heterologous combinations). While the mixture was incubating for 30 min at 37°C, a carbon-coated support grid was introduced into a conical centrifuge tube containing ammonium acetate solution, such that it sank to the bottom, carbon side up. One drop of incubated bacteria and antiserum mixture was sucked into a Pasteur pipette with a little ammonium acetate solution, and this was introduced into the top of the centrifuge tube and mixed as near to the top as possible. The tube was then centrifuged at about 1000 g for 10 min, the ammonium acetate was pipetted off, and the grid was removed by sliding it up the side of the tube with a bent wire. After three washes in ammonium acetate it was negatively stained with 1% (w/v) sodium phosphotungstate solution. This method differs from that of Lawn & Meynell (1970) in the mounting procedure only, and the results should be comparable with theirs.

Antiserum against F pili was kindly supplied by Dr E. Meynell and had been prepared using whole cells (Lawn & Meynell, 1970).

RESULTS

Occurrence of 711 pili

A preliminary search for pili determined by the four plasmids currently assigned to the X incompatibility group (R6K, R485, R711b, R778b) was made in the electron microscope using bacteria from streaks on plates containing appropriate selective drugs. While the
background strain, *E. coli* 153, possessed type I pili (Brinton, 1965), with the R+ strains used here these appendages only occurred on a very small proportion of cells (about 5%) from both plate and broth cultures. In a sample of 100 cells from a plate, 93 had no pili, 6 had one pilus and 1 had 39 pili. The presence of other filaments with a different morphology to type I pili (short, very straight, often broken in half) was therefore easily detectable. Plasmids R6K and R485 (Hedges et al., 1973) did not determine any filaments that could be distinguished from type I pili and so they were not investigated further. With R711b and

Fig. 1. A cell of *E. coli* 153-1(R778b) with pili. From a broth culture (5 h shaking, 30 min static). A single type I pilus is arrowed.

Fig. 2. A type I pilus (top) and a 711 pilus (bottom) from a plate culture of *E. coli* 153-2(R711b). The short curved filament (bottom centre) is a λ phage tail. 153 is lysogenic for λ.
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Table 2. Numbers of pili determined by various plasmids in strains of E. coli and S. typhimurium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pilus type</th>
<th>Pili/cell</th>
<th>Cells piliated (%)</th>
<th>Pili/piliated cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli J53-2(R711b)†</td>
<td>711</td>
<td>2.80</td>
<td>86</td>
<td>3.26</td>
</tr>
<tr>
<td>S. typhimurium SQ1139(R711b)§</td>
<td>711</td>
<td>0.02</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>E. coli J53-1(R778b)†</td>
<td>711</td>
<td>2.22</td>
<td>56</td>
<td>3.96</td>
</tr>
<tr>
<td>S. typhimurium SQ1139(R778b)§</td>
<td>711</td>
<td>0.04</td>
<td>4</td>
<td>1.00</td>
</tr>
<tr>
<td>E. coli CR34(F+)</td>
<td>F</td>
<td>1.00</td>
<td>66</td>
<td>1.50</td>
</tr>
<tr>
<td>E. coli C600(F′lac)†</td>
<td>F′lac</td>
<td>2.16</td>
<td>64</td>
<td>3.38</td>
</tr>
</tbody>
</table>

* Percentage of piliated cells in a sample of 50 cells.
† Average number of pili per pilus-bearing cell in a sample of 50 cells.
‡ Type I pili were present, but conjugative pili could be clearly distinguished for counting by their thickness (Fig. 2).
§ Several pili were seen on clumps of cells but were not in the sample counted.

Table 3. Stability of the plasmids R711b and R778b in various background strains

Portions (0.1 ml) of 10⁻⁴ dilutions of 5 h shake cultures in nutrient broth were spread on 'normal' plates, containing nutrients only, and on 'selective' plates, containing appropriate drugs (see Methods). Stability is expressed as the ratio of the number of colonies on selective plates to the number on normal plates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of colonies on plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>E. coli J53-2(R711b)</td>
<td>105</td>
</tr>
<tr>
<td>S. typhimurium SQ1139(R711b)</td>
<td>29</td>
</tr>
<tr>
<td>E. coli J53-1(R778b)</td>
<td>117</td>
</tr>
<tr>
<td>S. typhimurium SQ1139(R778b)</td>
<td>40</td>
</tr>
</tbody>
</table>

R778b, however, cells carrying the plasmids bore long filaments having a morphological resemblance to F pili (Fig. 1) and about twice as thick as type I pili (Fig. 2).

In order to establish unequivocally that the pili were determined by R711b and R778b, the two plasmids were transferred to S. typhimurium which bore no type I pili. Bacteria from both donors and recipients without selective drugs were examined in the electron microscope and the numbers of pili/cell were determined (Table 2). Pilus counts for strains of E. coli with F and F′lac are included in Table 2 for comparison. There were more 711 and F′lac pili present in strains of E. coli than F pili. With strains of S. typhimurium, however, the numbers of 711 pili were much lower (although more were obtained from selective plate cultures). Thus when R711b and R778b are introduced into different bacterial genera, morphologically similar pili are determined.

Stability of R711b and R778b

As noted above, 711 pili are only expressed at a low frequency in S. typhimurium SQ1139 when no selective drugs are present. To determine whether or not this might be due to the build-up of spontaneous R⁻ segregants (bacteria which had lost their plasmids), the stabilities of R711b and R778b were compared in E. coli and S. typhimurium in liquid culture. Dilutions of 5 h shake cultures in nutrient broth without selective antibiotics were spread on plates with and without appropriate drugs. The results (Table 3) show that both R711b and R778b are stable in strains of E. coli but very unstable in S. typhimurium SQ1139. Plasmid instability is thus the most likely explanation for the low piliation of the two S. typhimurium strains.
Fig. 3. 711 pili from a plate culture of E. coli 353-1(R778b) showing a dark axial line, and what is believed to be the pilus base (centre).
Fig. 4. The centre of Fig. 3 enlarged at higher contrast.

Dimensions and morphology of 711 pili

There is a marked tendency for F pili to form aggregates in liquid suspension after removal from cells (Brinton, 1971). 711 pili do not have this property and isolated filaments lie at random angles on carbon support films (Fig. 3). F pili lie in parallel clusters with relatively few discrete filaments. Suspensions of F_{Olac} pili also form aggregates (not illustrated).

No difference in thickness between F and 711 pili could be detected in the electron microscope. Direct measurements from negatively stained preparations gave a diameter of about 9.5 nm for 711 pili. The generally accepted diameter of F pili is 9.0 nm; Brinton (1965) gives 8.5 nm, and Lawn (1966) 9.5 nm. F_{Olac} pili appear to be about the same thickness.
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Fig. 5. A 711 pilus from a plate culture of E. coli J53-2(R711b) showing a fragment of cell envelope attached to one end (top).

Fig. 6. An F pilus labelled with RNA phage R17, and a 711 pilus with no adsorbed virions. R17 suspension was mixed with cells of E. coli CR34(F+) and J53-2(R711b) and incubated for 10 min before mounting for electron microscopy.

Measurements of the length of 74 F pili and 82 711 pili gave arithmetic means of 2400 nm and 2100 nm respectively. F pili had a range of about 150 to 10500 nm and 711 pili 50 to 10250 nm. Modal lengths were slightly different: 1500 nm for F pili and 1200 nm for 711 pilus. The overall differences in these measurements are relatively small and are not considered significant.
The general appearance of 711 pili closely resembles that of F pili. Some filaments exhibited a clear dark line down their axis (Fig. 3). Pili which had become detached from cells often had one end either thickened (Fig. 3 centre, Fig. 4) or with a fragment of membrane attached (Fig. 5). The latter sometimes resembled the knobs of F pili (Lawn, 1966). The following preparations were examined to determine whether these structures were located at the distal or proximal ends of the pili. In a broth culture of 153-2(R711b) (5 h with shaking, 30 min static), virtually all pili remained attached to the cells, as shown for 153-1-(R778b) in Fig. 1; a sample of 50 pili bore no terminal structures. However, in a sample of 50 detached pili from a plate culture, 11 had thickened ends, 21 had membrane fragments of various sizes and 18 had no terminal structures. The inference is that the terminal structures are at the pilus bases rather than the tips.

As confirmation, adsorbed filamentous phage fd was used to mark the pilus tips (distal ends, see below) and the free ends were scored for terminal structures. This was done by mixing a cell-free pilus suspension in broth (see Methods) with high titre fd, and incubating for 10 min at 37 °C. In a sample of 50 marked pili, 12 had thickened ends, 16 had fragments of membrane attached and 22 had no terminal structures. These figures are close to those obtained without adsorbed fd and the terminal structures are therefore located at the pilus base.

The structure described as a thickened end has the appearance of an open-ended tube (Fig. 4), and probably represents the point of assembly of the pilus. The membrane fragments were doubtless torn from the cell envelope when the pili were sheared off during preparation. It is not clear whether the knobs of F pili are the same as the membrane fragments; Lawn & Meynell (1970) identified some as cell envelope material (using immune electron microscopy) and some as pilin (pilus protein). Possibly the former are located at the bases of F pili, and the latter at the tips. It is beyond the scope of this paper to examine the question of the knobs of F and Folac pili, which also have them.

**Bacteriophage f711**

Phage f711 was isolated by transferring R711b to a strain of *S. typhimurium* which had been made resistant to most of the bacteriophages (not plasmid-specific) commonly found in sewage, and using this to isolate an R711b-specific phage by enrichment (see Methods). The host range of f711 (see below) suggested that it might be similar to the F-specific filamentous phages. This was supported by its length (8700 nm based on polystyrene latex) and thickness (about 6 nm), which are similar to the dimensions of the F-specific phages. Since fd (Marvin & Hoffmann-Berling, 1963) is a fully characterized bacterial virus, this was used for most of the following experiments, f711 being included only when a comparison seemed desirable.

**Sensitivity to bacteriophages of strains carrying various plasmids**

Phage sensitivities for strains carrying R711b, R778b, F and Folac were determined initially by the spot test (Table 4). None of the strains tested was sensitive to the R-specific filamentous phages IKe (IncN) and Pf3 (IncP-1), or to the lipid phage PR4 (IncN, IncP-1, IncW). While a negative result with the spot test does not rule out a low level of sensitivity, this was not considered likely since no adsorption of these phages to pili or cell surfaces was observed in the electron microscope. The F-specific RNA phages ZIK/1 (Bradley, 1964), which is similar to Qβ (Watanabe, 1964), and R17 (Paranchych & Graham, 1962) gave clearing only with their host, *E. coli* cr34(F+). Again, electron microscopy revealed no adsorption to 711 or Folac pili (see below) so that the negative spot tests were taken as indicating complete resistance. The filamentous phages fd and f711 showed clearing with strains of *E. coli* with each of the plasmids carried [*E. coli* c600(Folac) showed only slight clearing], but neither of the strains of *S. typhimurium* showed any clearing. A titre increase test was therefore carried out with phage fd (see Methods): the increase was 1.38 × for
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Table 4. Susceptibility to bacteriophages of strains carrying plasmids as indicated by the spot test

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bacteriophage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZIK/I</td>
</tr>
<tr>
<td>E. coli 153-2(R711b)</td>
<td></td>
</tr>
<tr>
<td>S. typhimurium SQ1139(R711b)</td>
<td></td>
</tr>
<tr>
<td>E. coli 153-1(R778b)</td>
<td></td>
</tr>
<tr>
<td>S. typhimurium SQ1139(R778b)</td>
<td></td>
</tr>
<tr>
<td>E. coli cr34(F+)</td>
<td></td>
</tr>
<tr>
<td>E. coli c600(F0lac)</td>
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</table>

+, Discernible clearing; −, no clearing. [The clarity of + spots was more or less similar except those for c600(F0lac) (fd, f711) which were very turbid.]

S. typhimurium SQ1139(R711b) for a period of 24 h, and 1·55× for SQ1139(R778b). Although small, it is considered that these increases indicate the sensitivity of both strains to fd. It is notable that f711, which appears similar to fd, was isolated on S. typhimurium SQ1139 D3(R711b), supporting the conclusion that sensitivity to the filamentous phages is conferred by R711b and R778b. The overgrowth of R- segregants probably prevented the production of more fd (see above), but the possibility of restriction cannot be ruled out.

The plaques formed by fd and f711 on E. coli cr34(F+) were clearest and the best defined [size about 2 mm in 0·5% (w/v) soft agar]. Escherichia coli 153-2(R711b) gave smaller plaques (about 1 mm), and 153-1(R778b) larger but more turbid ones (about 3 mm). With E. coli c600(F0lac), clearly distinguishable plaques could not be obtained although the spot test definitely indicated sensitivity to the phages. Likewise, no plaques were obtained with S. typhimurium SQ1139(R711b) and SQ1139(R778b) though the titre increase test indicated sensitivity.

Adsorption of F-specific bacteriophages to 711 pili

The resistance of strains carrying R711b and R778b to the F-specific RNA phages was due to the inability of 711 pili to adsorb them (Fig. 6). RNA phages attach to the sides of F pili, but F-specific filamentous phages attach to the tips (Caro & Schnös, 1966). In order to ascertain whether or not f711 attached to 711 pili in the same way, E. coli 153-2(R711b) from a plate culture was suspended in a preparation of f711 (1×1011 p.f.u. ml⁻¹), incubated for 10 min and examined in the electron microscope. Many of the 711 pili which had become detached from the cells bore pairs of f711 virions (Fig. 7); some had one and very few had three. None of the pili attached to the cells had any adsorbed phages. The F-specific filamentous phage fd behaved in the same way. These observations are generally in accord with those reported for the adsorption of F-specific filamentous phages to F+ cells (Marvin & Hohn, 1969; Jacobson, 1972), save for the preferred multiple adsorption of f711 and fd to 711 pilus tips. Caro & Schnös (1966) found 18 F pili (or fragments) with two f1 filamentous phages attached in a sample of 142 pili and fragments; 100 had one virion each, 1 had three virions, and the remainder none. This aspect was studied in more detail as follows.

Because RNA phages do not adsorb to the sides of 711 pili, these small virions cannot be used for marking the pili to distinguish them from filamentous phages. However, the difference in thickness and appearance is adequate for this purpose (Fig. 7). A suspension of 711 or F pili (as used to inoculate rabbits, see Methods) was mixed with phage fd at 1×1015 p.f.u. ml⁻¹ and incubated at 37 °C for 15 min. Grids for electron microscopy were floated for about 1 min and then negatively stained in 2% (w/v) sodium phosphotungstate solution. Pili were scored at random as bearing zero, one or two virions (no examples with three were found in this experiment). The ratio of fd:pili was estimated approximately by counting large numbers of the two kinds of filament in random areas in the electron microscope either directly or from micrographs. The very few type I pili were easily distinguished...
Fig. 7. Phage f711 adsorbed in pairs to one end of free 711 pili. Cells of *E. coli* 153-2(R711b) from a plate culture were mixed with a suspension of f711 and incubated for 10 min. 

from fd; the former were straight and short, and the latter curved and of uniform length. Two determinations were carried out for each type of pilus using different fd:pili ratios. The results (Table 5) must be treated qualitatively since the fd:pili ratio could not be determined accurately, nor could it be controlled adequately to give the same value for each type of pilus. Even with these limitations it is clear that 711 pili adsorb fd much more efficiently than F pili and that there are more pairs of fd adsorbed to 711 pili than single
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Table 5. Numbers of virions of bacteriophage fd adsorbed to free F and 711 pili from strains of E. coli

Pilus preparations (see Methods) contained a number of fragmented pili. These can adsorb filamentous phages to one end as efficiently as intact pili (Caro & Schnös, 1966), therefore all fragments were scored. Very few type I pili were present and were easily identified.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sample size (pili)</th>
<th>Ratio fd:pili*</th>
<th>Percentage of pili with no. of phages adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR34(F+)</td>
<td>69</td>
<td>10</td>
<td>95.5 4.5 0</td>
</tr>
<tr>
<td>J53-2(R711b)</td>
<td>127</td>
<td>5</td>
<td>61.5 10.0 28.5</td>
</tr>
<tr>
<td>CR34(F+)</td>
<td>135</td>
<td>4</td>
<td>95.6 3.7 0.7</td>
</tr>
<tr>
<td>J53-2(R711b)</td>
<td>150</td>
<td>2.5</td>
<td>77.3 10.7 12.0</td>
</tr>
</tbody>
</table>

* Approximate value only, quoted to nearest 0.5 (see text).

No fd virions could be found absorbed to F6lac pili examined in the same way. Caro & Schnös (1966) obtained a higher incidence of fI adsorbed to free F pili, but they were using different conditions (media, probably a higher concentration of interacting elements etc.) so their values are not comparable.

The more efficient adsorption of fd by 711 pili compared with F pili should be reflected in the adsorption of the bacteriophage by bacteria in the exponential phase of growth. The efficiencies of adsorption were therefore determined: E. coli CR34(F+) adsorbed 22.4% of the phage and E. coli J53-2(R711b) 31.9%. This difference is considered significant. The greater adsorption efficiency of the latter probably reflects both the higher level of piliation compared with E. coli CR34(F+) and the greater affinity of 711 pilus tips for fd, compared with F pilus tips.

**Immune electron microscopy**

The serological tests were carried out with three objectives: (i) to demonstrate that the pili transferred at the same time as the drug resistance markers of R711b were serologically similar to those on the donor strain; (ii) to determine whether or not the pili on strains carrying R711b were serologically similar to those on stains carrying R778b; and (iii) to ascertain whether or not there was any serological relationship between F, F6lac and 711 pili. A preliminary test was carried out using the grid labelling method (Lawn, 1967). This essentially provides a positive or negative result indicating that the pili are either serologically identical (shown by heavy labelling with antibodies on both homologous and heterologous pili) or not serologically identical (heavy labelling on homologous pili but no apparent labelling on heterologous pili). Negative staining is usually too poor to distinguish small numbers of adsorbed antibodies which would indicate a limited serological relationship (Lawn & Meynell, 1970). The results (Table 6) show that antiserum against 711 pili labels all the pili except F and F6lac pili. In test 1, E. coli J53-2(R711b) was used as the donor in constructing S. typhimurium SQ1139(R711b), so that the pili *transferred* are serologically identical. Tests 2, 3 and 4 show that the pili determined by R711b are serologically identical to those determined by R778b. However, in tests 5 and 6 no labelling was observed suggesting that F and F6lac pili might be serologically different to 711 pili. To determine whether this difference was partial or complete, a second series of tests was carried out using the suspension labelling method. With this method, better negative staining allows the detection of small numbers of adsorbed antibodies. However, only heavily coated pili, or pili with no antibodies at all could be found; there were no intermediate relationships in the combinations of pilus type and antiserum tested (Table 7). The results obtained with anti-J53-1(R778b) pili confirmed those with anti-J53-2(R711b) pili. Those obtained with anti-22(F+) were confirmed (results not shown) using a different sample of antiserum to F pili (supplied by Dr E. Meynell).
D. E. BRADLEY

Table 6. Antibody labelling of 711, F and F6lac pili by the grid method

Bacteria were treated with antiserum after mounting on the grid.

<table>
<thead>
<tr>
<th>Test</th>
<th>Pili on strain</th>
<th>Antiserum*</th>
<th>Reaction†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. typhimurium SQ1139(R711b)</td>
<td>532(R711b)</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>E. coli 153-1(R778b)</td>
<td>532(R711b)</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>E. coli 153-2(R711b)</td>
<td>532(R778b)</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>S. typhimurium SQ1139(R778b)</td>
<td>532(R711b)</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>E. coli cr34(F+)</td>
<td>532(R711b)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>E. coli c600(F6lac)</td>
<td>532(R711b)</td>
<td></td>
</tr>
</tbody>
</table>

* Strain of E. coli from which pilus preparation used as antigen was made.
† +, Pili heavily coated with antibodies; −, uncoated pili.

Table 7. Antibody labelling of 711, F and F6lac pili in suspension

Dilutions used for homologous antisera (giving +) were 1 in 20, and for heterologous antisera (giving −) 1 in 5, except for 153-2(R711b) plus anti-153-2(R778b) the dilution was 1 in 10.

<table>
<thead>
<tr>
<th>Antiserum*</th>
<th>Pili on strain</th>
<th>22(F+)</th>
<th>153-2(R711b)</th>
<th>153-2(R778b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 153-2(R711b)</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>E. coli cr34(F+)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>E. coli c600(F6lac)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

+, Pili heavily coated with antibodies; −, no detectable antibodies.

* Strains of E. coli from which pilus preparation used as antigen was made. Anti-22(F+) was kindly supplied by Dr E. Meynell.

Electron micrographs of some representative combinations of antisera and pilus type are shown in Figs 8 to 15. Figures 8 and 9 demonstrate that pili determined by R711b and R778b are serologically similar and that 711 pili can be transferred to S. typhimurium. Figures 10 to 15 are arranged in the same way as columns 2 and 3 in Table 7 with the same combinations. The electron micrographs are typical and show that there is no serological relationship between F, F6lac and 711 pili. The lack of adsorbed antibodies on pilus tips with heterologous combinations (Figs 10, 13, 14 and 15) does not necessarily mean that a serologically related structure is absent on the different pili; pilus tip antibodies might well be present at too low a concentration to provide detectable adsorption. A common ability to adsorb the F-specific filamentous phages suggests some similarity between the tips, though another explanation is possible (see Discussion). In Fig. 15, the appearance of the pilus ends suggests that F6lac pili may have an open-ended structure like 711 pili (Fig. 4).

DISCUSSION

This paper describes a new type of pilus determined by two plasmids originating from widely separated localities. R711b was found in a Providence strain from Birmingham, England, and R778b in another from Winnipeg, Canada (Hedges, 1974). 711 pili resemble F and F6lac pili in appearance, length, thickness and in a common ability to adsorb F-specific filamentous bacteriophages, but they differ in that 711 pili (and F6lac pili) cannot adsorb F-specific RNA phages. 711 pili are serologically unrelated to those of F and F6lac.

One of the features of 711 pili is their preferred tendency to adsorb two F-specific filamentous phage virions at their tips. F pili generally adsorb only one (Caro & Schnös, 1966) though some attached pairs can be found. This suggests that there may be two separate adsorption sites at the 711 pilus tip, or that there may simply be space available for two virions to fit on the exposed receptor protein, although the likelihood of a more complex explanation,
Conjugative pili of plasmids R711b and R778b

Fig. 8. Pili from *S. typhimurium* sq1139(R778b) labelled with antiserum to *E. coli* 153-2(R711b). Grid method.

Fig. 9. Pili from *E. coli* 153-2(R711b) labelled with antiserum to *E. coli* 153-1(R778b). Grid method. The pili are almost obscured by the antibodies.

Fig. 10. Pili from *E. coli* 153-2(R711b) labelled in suspension with antiserum to *E. coli* 22(F') diluted 1 in 5, showing no adsorbed antibodies.

Fig. 11. Pili from *E. coli* 153-2(R711b) labelled with homologous antiserum diluted 1 in 10, suspension method.

Fig. 12. Pili from *E. coli* cr34(F') labelled with antiserum to *E. coli* 22(F') diluted 1 in 20, suspension method.

Fig. 13. Pili from *E. coli* cr34(F') labelled with antiserum to *E. coli* 153-2(R711b) diluted 1 in 5, suspension method. There are no adsorbed antibodies.

Fig. 14. Pili from *E. coli* c600(Fgal) labelled with antiserum to *E. coli* 22(F') diluted 1 in 5, suspension method. There are no adsorbed antibodies.

Fig. 15. Pili from *E. coli* c600(Fgal) labelled with antiserum to *E. coli* 153-2(R711b) diluted 1 in 5, suspension method. There are no adsorbed antibodies.
perhaps involving allosteric changes in the pilus tip, must also be considered. With 711 pili, the attachment of the first phage could cause a change in the receptor protein providing favourable conditions for the attachment of a second virion. If the adsorption of the first phage was reversible, a second phage would be required for irreversible attachment and infection. This is contrary to the conclusion drawn by Caro & Schnös (1966) who considered that the adsorption of a phage to an F pilus tip interfered with the adsorption of a second one. In any case, a difference in the adsorption characteristics of F and 711 pili is indicated. With F_\text{l}_{a}c pili the lack of filamentous phage adsorption noted in the electron microscope was probably related to the inability of fd to form plaques on E. coli c600(F_\text{l}_{a}c).

Immune electron microscopy demonstrated that the filaments designated 711 pili are determined by both R711b and R778b. That 711 pili are serologically unrelated to F pili is not surprising since 711 pili cannot adsorb F-specific RNA phages. 711 pili and F_\text{l}_{a}c pili are also serologically unrelated in spite of a common inability to adsorb RNA phages. In addition, F and F_\text{l}_{a}c pili have been found to be serologically unrelated contrary to previous observations (Meynell, Meynell & Datta, 1968). As well as the serological difference between 711 and F_\text{l}_{a}c pili, the latter tend to aggregate longitudinally into bundles but 711 pili lack this property. A difference in the pilin is therefore implied. Furthermore, Datta (personal communication) has shown that there is no incompatibility between R711b or R778b and F_\text{l}_{a}c. While these factors suggest a genetic difference, further studies beyond the scope of this paper are required to determine where similarities, if any, may lie.

The ability of the filamentous phages to infect strains carrying F, F_\text{l}_{a}c or 711 pili is of particular interest. The filamentous phages could well be non-specific like PR4, a lipid-containing virus adsorbing to the tips of very different pilus determined by IncW and IncP-1 plasmids (Bradley, 1976; Bradley & Cohen, 1977). This is contrary to the proposal that filamentous phages are specific for a single type of pilus, or possibly another type of receptor in the case of the IncN phage IKe (Brodt, Leggett & Iyer, 1974).

Note added in proof. Since going to press, it has been found that the pili borne by the strain described here (see Methods) as Escherichia coli c600(F_\text{l}_{a}c) are serologically different from those borne by the original Salmonella typhi (F_\text{l}_{a}c) supplied by Dr S. Falkow (see Falkow & Baron, 1962). It is therefore evident that the plasmid used to represent F_\text{l}_{a}c in this work is not identical with the original. However, tests have been carried out on the pili of S. typhi (F_\text{l}_{a}c), and it has been found that, with two exceptions, the results are the same as those described here for E. coli c600 (F_\text{l}_{a}c) pili. The exceptions are, first, the original F_\text{l}_{a}c pili do not aggregate into longitudinal bundles like those of E. coli c600 (F_\text{l}_{a}c) and, second, the number of pili/cell has not been estimated for a strain of E. coli carrying the original F_\text{l}_{a}c plasmid. Clearly the differences between the two plasmids should be investigated.

I am most grateful to Doris Cohen for valuable technical assistance, which included the preparation of pilus suspensions and antisera. Detailed correspondence with Drs N. Datta and W. Paranchych was much appreciated as also were the gifts of strains shown in Table 1, and antisera from Dr E. Meynell. Dr Barry Hall of this University kindly provided phages fd and R17. The work was supported by the Medical Research Council of Canada, grant no. MA5608.
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