Serological Characteristics of Pili Determined by the Plasmids R711b and F₀lac

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The plasmids R711b (at present IncX) and F₀lac (IncFV) both determine pili morphologically like those of F (IncFI), and confer sensitivity to the F-specific filamentous bacteriophages, but not to the F-specific isometric RNA phages. Detailed serological studies show that the two pilus types are unrelated, and that neither is related to any of the previously defined F pilus serotypes. Adsorption of the isometric RNA phage MS2 to R711b pili occurs in the presence but not in the absence of formalin, which presumably prevents elution of reversibly adsorbed virions. No adsorption occurs with F₀lac pili. MS2 multiplication, as measured by titre increase tests in liquid medium, is found with neither plasmid. The two plasmids are not incompatible. These observations indicate that R711b and F₀lac are different both from one another and from the plasmids belonging to the incompatibility groups IncFI–IV.

INTRODUCTION

The plasmids R711b and R778b, assigned to incompatibility group X (IncX), have been found (Bradley, 1977) to determine pili (designated 711 pili) which are morphologically similar to those of F and are able to promote infection by F-specific filamentous bacteriophages, which use the end of the pilus as receptor, but not F-specific RNA phages, whose receptors lie in the pilus sides. Since these are the pilus characteristics originally noted for the *lac*⁺-transferring IncFV plasmid F₀lac (Falkow & Baron, 1962; Brinton, 1965; Lawn *et al.*, 1967; Meynell *et al.*, 1968; Datta, 1975), F₀lac pili have now been re-examined so that they may be included in the present survey, and so that previous results (Bradley, 1977, note added in proof) may be confirmed. The serological relationships of 711 and F₀lac pili with one another and with the four F pilus serotypes (Lawn & Meynell, 1970) have therefore been investigated in detail, together with their affinities for F-specific RNA phages, and the incompatibility relationships between the plasmids.

Most of the present experiments have been carried out with a derepressed (*drd*) version of F₀lac, constructed by N. Willetts, which determines large numbers of pili. It will be demonstrated that these are identical with the pili found on the original *Salmonella typhi* (F₀-*lac*⁺) strain described by Falkow & Baron (1962). To avoid confusion, the plasmid F₀-*lac*⁺ will be referred to by its later designation, F₀lac (Datta, 1975), and F₀lac *drd* by the designation of Willetts: EDP208.

Table 1. *Bacterial strains used*

Strains with plasmids	Incompatibility group	Synonym and description*	Source and/or reference
<i>Salmonella typhi</i> (F ₀ lac)	FV	ST-2, original strain, Pil ⁺	S. Falkow (Falkow & Baron, 1962)
<i>Escherichia coli</i> 24(F ₀ lac)	FV	rc24, Thr ⁻ , Leu ⁻ , Thi ⁻ , Sm ^r , Pil ⁻	N. Datta (Datta <i>et al.</i> , 1966)
<i>E. coli</i> jc6256(EDP208)	FV	jc6256 background strain is lacΔX74 and Trp ⁻	N. Willetts
<i>E. coli</i> j53-2(R711b)	X	Pro ⁻ , Met ⁻ , Rif ^r , Km ^r , Pil ⁺	N. Datta (Hedges, 1974)
<i>E. coli</i> j62(R711b)	X	Pro ⁻ , His ⁻ , Trp ⁻ , Lac ⁻ , Km ^r , Pil ⁺	N. Datta (Hedges, 1974)
<i>E. coli</i> CR34-1(F ⁺)†	FI	Thr ⁻ , Leu ⁻ , Thi ⁻ , Thy ⁻ , LacY ⁻ , Nal ^r , Pil ⁻	Author
Recipient strains			
<i>E. coli</i> CR34(sm-r)†		Thr ⁻ , Leu ⁻ , Thi ⁻ , Thy ⁻ , LacY ⁻ , Sm ^r , Pil ⁻	Author
<i>E. coli</i> CR34-1†		As CR34-1(F ⁺)	Author
<i>E. coli</i> CR34-2†		CR34-3(rif-r), Thr ⁻ , Leu ⁻ , Thi ⁻ , Thy ⁻ , LacY ⁻ , Rif ^r , Pil ⁻	Author (Bradley & Cohen, 1976)

* Abbreviations: Pil⁺, type I pili present; Pil⁻, type I pili absent; drug resistance markers as in Novick *et al.* (1976); auxotrophic markers as in Bachmann *et al.* (1976).

† All CR 34 strains originated from an R⁻ segregant of CR34(R1822), which was described by Olsen & Shipley (1973). Numbering for drug resistance has been changed to the system used by Datta.

METHODS

Bacterial strains, bacteriophages and growth media. The strains used are listed in Table 1. *Salmonella typhi* (F₀lac) was supplied in lyophilized form dated 1960. *Escherichia coli* 24(F₀lac) was originally obtained by N. Datta from K. Sanderson, who, it is understood, obtained it from L. Baron. The repressed F₀lac plasmid used by N. Willetts to construct the derepressed mutant EDP208 (F₀lac *drd*) was obtained from E. S. Anderson as strain 42R328.

The RNA-containing F-specific bacteriophage used was MS2 (Davis *et al.*, 1961).

Oxid Nutrient Broth or BBL Brain Heart Infusion Broth, with 2% (w/v) agar added for plates, were used as routine growth media. M9 minimal medium (NH₄Cl, 1 g l⁻¹; KH₂PO₄, 3 g l⁻¹; Na₂HPO₄, 6 g l⁻¹) was supplemented with lactose (0.25%, w/v), MgSO₄ (0.5 mM), and other additives as appropriate to nutritional requirements. BBL MacConkey Agar (abbreviated as MC) was used where an indication of lactose fermentation was required. All incubation was at 37 °C.

Matings. All matings were carried out by standard methods and were for 1 h with equal numbers of donor and recipient cells. Counterselection of the donor was with streptomycin (200 µg ml⁻¹) or rifampicin (100 µg ml⁻¹) or nalidixic acid (25 µg ml⁻¹). For R711b, plasmid selection was with kanamycin (20 µg ml⁻¹). For F₀lac and EDP208, transfer from *S. typhi* (F₀lac) and *E. coli* jc6256(EDP208) to *E. coli* CR34-2 was achieved by spreading 0.3 ml of a suitable dilution of mating mixture on 15 cm diam. plates (MC agar plus nalidixic acid), selecting for recipients only. Lac⁺ colonies were selected visually and purified. Due to a low transfer frequency, F₀lac required an overnight mating to produce sufficient numbers of Lac⁺ colonies. Strain CR34-2(F₀lac) was checked for its ability to transfer *lac* by mating with CR34-1. Strain CR34-2(EDP208) was checked for the production of pili reacting with antibodies to jc6256(EDP208) pili. Strain CR34(sm-r)(R711b) was checked for 711 pili which reacted with antibodies to *E. coli* j53-2(R711b) pili. Fewer 711 pili were produced than by j53-2(R711b), which was therefore used for quantitative tests. For incompatibility testing, strains carrying the two plasmids were constructed by transferring R711b from *E. coli* j62(R711b) to CR34-2 (F₀lac) and CR34-2(EDP208). The recipient strains were then tested for separate transfer of the two plasmid markers as described in the text.

Titre increase test. The method of Stanisich (1974) was used. Phage MS2 was added to early exponential phase shake cultures to a concentration of 2 × 10⁴ plaque forming units (p.f.u.) ml⁻¹. The cell-free culture fluids were titrated after a further 5 h incubation. Since R711b often proved unstable in CR34(sm-r), bacteria as used in the test were spread on nutrient plates with and without kanamycin, and the two colony counts were compared. Colonies on kanamycin numbered 95% of those on nutrient agar alone.

Efficiency of MS2 adsorption by bacterial strains. Cultures were shaken for 5 h then incubated statically to allow optimum production of 711 pili. MS2 was added to 2 × 10⁸ p.f.u. ml⁻¹, and after 15 min gentle shaking the cell-free culture fluids were titrated for surviving phage (see Bradley, 1977).

Antisera. Antisera to j53-2(R711b) and jc6256(EDP208) were produced by intravenous inoculation of

Table 2. *Reactions of 711 and EDP208 pili with their antisera*

Pili on strain:	Reaction* with antiserum to:	
	jc6256(EDP208)	j53-2(R711b)
<i>S. typhi</i> (F ₀ lac)†	+	—
<i>E. coli</i> jc6256(EDP208)	+	—
<i>E. coli</i> j53-2(R711b)	—	+

* Reaction levels are not graded since all positive results were at the + + + + level of Lawn & Meynell (1970). Thus, + indicates heavy labelling and — indicates none.

† Because of the very small numbers of pili determined by F₀lac, which is repressed, it was necessary to use the grid-labelling method for this strain (see Methods).

rabbits with pilus suspensions. These were prepared by homogenizing concentrated bacterial suspensions with a Thomas Teflon Pestle Tissue Grinder followed by partial purification using differential centrifugation (Bradley, 1977). Pilus production by the original F₀lac, being repressed, was too low for use in the preparation of antiserum. Antisera to the various F pilus serotypes were prepared using whole cells (in some cases, formalized) instead of pilus suspensions (Lawn & Meynell, 1970). Antibacterial antibodies were removed by adsorption with host bacterium unless otherwise specified (see Results, Table 3). All antisera were used at a concentration which reacted with homologous pili at the + + + + level using the grading method of Lawn & Meynell (1970).

Electron microscopy. For pilus counts, bacteria were mounted on carbon-coated support grids as described elsewhere (Bradley, 1977) and negatively stained with 0.15 % (w/v) sodium phosphotungstate solution. Pili were counted on 50 to 100 isolated cells.

Pili were labelled with antibodies by suspending bacteria in diluted antiserum and incubating for 30 min. Cells were then mounted and negatively stained by two different procedures which gave comparable results (Lawn & Meynell, 1970; Bradley, 1977). Alternatively, with grid-labelling (Lawn, 1967), grids with bacteria on them were floated on diluted antiserum then negatively stained.

Tests for adsorption of phage MS2 were done in two ways. The first method used the addition of formalin to permit detection of reversible adsorption. Bacteria were mixed with MS2 at 2×10^{10} p.f.u. ml⁻¹, and after 15 min at room temperature formalin was added to a final concentration of 0.5 % (v/v). Formalin does not prevent MS2 adsorption. After standing at room temperature for 24 h, bacteria were mounted on specimen grids and negatively stained in 1 % (w/v) uranyl acetate solution. In the second method, bacteria from overnight plates were suspended to a high concentration in a drop of phage MS2 suspension at a concentration of 5×10^{11} p.f.u. ml⁻¹. After 10 min at 37 °C, cells were mounted for electron microscopy and negatively stained in 0.4 % (w/v) sodium phosphotungstate solution.

RESULTS

Antigenic relationships of pili determined by R711b, F₀lac and EDP208

Table 2 indicates the relationships between the pili of F₀lac, EDP208 and R711b. Figures 1 to 4 show that antiserum to EDP208 pili reacted as strongly with pili borne by *S. typhi* (F₀lac) and *E. coli* 24(F₀lac) as with the homologous EDP208 pili, but did not react with 711 pili on j53-2(R711b). Conversely, the pili borne by *S. typhi* (F₀lac) did not react (Fig. 5) with antiserum to 711 pili which gave a + + + + reaction with homologous pili (Bradley, 1977). Figure 6 illustrates a 'knob' (Lawn & Meynell, 1970) at the distal end of a pilus on 24(F₀lac), in which the plasmid is repressed. Strain 24(F₀lac) carried only 0.02 pili/cell and *S. typhi* (F₀lac) produced even fewer: only about 5 on an entire electron microscope grid. A high frequency of 'knobs' was found with the derepressed EDP208, which determines up to 20 pili/cell (Willetts, Moore & Paranchych, unpublished).

Reactions of 711 and EDP208 pili with antisera to the F pilus serotypes

Previous observations (Bradley, 1977) indicated that 711 pili were serologically different from F pili. The results in Table 3 extend this conclusion to the other F serotypes and were obtained using 711 pili on j53-2(R711b) and F₀lac pili on jc6256(EDP208). They show the

All bar markers represent 100 nm. All negative staining was with sodium phosphotungstate.

Fig. 1. Pili on a cell of *E. coli* CR34-2(EDP208) treated with antiserum to *E. coli* JC6256(EDP208) showing ++++ labelling by the suspension method.

Fig. 2. *Salmonella typhi* (F₀lac) pilus treated with antiserum to JC6256(EDP208) showing +++ to ++++ labelling by the grid method.

Fig. 3. *Escherichia coli* 24(F₀lac) pilus treated with antiserum to JC6256(EDP208) showing ++++ labelling by the grid method.

Fig. 4. 711 pili from *E. coli* J53-2(R711b) treated with antiserum to JC6256(EDP208) showing no reaction by the grid method.

All bar markers represent 100 nm.

Fig. 5. A pilus (thicker fragments are flagella) from *S. typhi* (F₀lac) treated with antiserum to *E. coli* j53-2(R711b), which gave + + + labelling with 711 pili (not illustrated). There are no visible antibodies (grid method, sodium phosphotungstate negative stain).

Fig. 6. Unlabelled F₀lac pilus from *E. coli* 24(F₀lac) showing terminal 'knob' (sodium phosphotungstate negative stain).

Fig. 7. F₀lac pilus from *E. coli* sc6256(EDP208) treated with unadsorbed anti-F serum (see Table 3) by the suspension method (uranyl acetate negative stain), showing the pilus tip with a few adsorbed antibodies (right of figure).

Fig. 8. 711 pili from j53-2(R711b) treated with host-adsorbed anti-R1 serum (see Table 3) by the suspension method (uranyl acetate negative stain), showing no adsorbed antibodies. Antibodies to bacteriophage λ were present in the antiserum; a λ tail is heavily coated.

Table 3. *Reactions of 711 and EDP208 pili with antisera to the four serotypes of F pili*

Serotype	Antiserum to plasmid:	Incompatibility group of plasmid†	Adsorbed with:‡	Reaction* with pili of:	
				R711b	EDP208
F	F	FI	— (unadsorbed)	NT	—
	F	FI	Host	—	NT
	F ₀ lac§	FI	Strain ED2118	—	NT
	F	FI	R1drd16	NT	—
R538F	R538Fdrd1	FIV	Host	—	NT
	R538Fdrd1	FIV	F and R1drd16	NT	—
R1	R1drd19	FII	Host	—	—
	R1drd19	FII	F	NT	—
R100	R192	FII	Host	—	—
	R192	FII	R538F	NT	—
	R192	FII	F and R1	NT	—

* The results refer to the sides of the pili: —, no reaction; NT, not tested. The tips of EDP208 pili were occasionally labelled (see text).

† The IncFIII representative ColB-K98 is not included since it determines R1-type pili (see text).

‡ Where 'host' is shown, antiserum was adsorbed with bacteria not carrying the plasmid.

§ Derivative of F₀lac, not F₀lac.

|| *Escherichia coli* strain ED2118 was used in place of the host for adsorption purposes.

reactions of F, R1, R538F and R100 antisera with the pilus sides, all of which were negative (Figs 7, 8). Occasionally ends of EDP208 pili did, however, show adsorbed antibodies with a particular anti-R1 serum adsorbed with host bacteria, and an unadsorbed anti-F serum (Fig. 7). The proportion of labelled tips was too small for the reaction to be readily explained in terms of an antigenic relationship limited to the pilus ends. Perhaps the particular ends reacting with the unadsorbed anti-F serum comprised attached cell wall fragments able to react with somatic antibodies, which had not been removed. Some 'knobs' on F pili evidently consist of cell wall material (Lawn & Meynell, 1970). By their failure to react with any of the antisera in Table 3, 711 and F₀lac pili also showed themselves to differ from ColB-K98 (IncFIII) pili, which are similar to those of R1, and ColB-K30 pili, which share antigens with F, R1 and R538F pili (see Meynell, 1978).

Adsorption of F-specific RNA-containing bacteriophages by 711 and EDP208 pili

Bacteria carrying the derepressed plasmid EDP208 are sensitive to the F-specific filamentous bacteriophages but resistant to the isometric RNA phages. Both R711b and F₀lac are similar in this respect (Lawn *et al.*, 1967; Bradley, 1977). To test for the adsorption of RNA phage MS2 to 711 and EDP208 pili, bacteria exposed to high concentrations of phage with and without formalin fixation were examined by electron microscopy. Without fixative, neither type of pilus showed any adsorbed virions (Fig. 9; 711 pili not illustrated). With fixation, however, 711 pili but not EDP208 pili were thickly coated (Fig. 10).

The efficiency of adsorption of MS2 by strains carrying R711b and EDP208 was also measured by assaying the numbers of p.f.u. remaining free after 15 min incubation of mixtures of bacteria and phage at a cell:phage ratio of 500:1 (see Methods). No adsorption could be detected, nor could any increase in MS2 titre in cultures of strains carrying R711b or EDP208 be demonstrated (Table 4). We therefore conclude that the adsorption of MS2 to 711 pili is of a reversible kind not leading to any significant level of infection; it is only detectable in the presence of formalin which stabilizes it. Both 711 and F₀lac pili are thus different from the other F pilus serotypes, which are functional receptors for F-specific RNA phages (Paranchych, 1975).

Bar markers represent 100 nm.

Fig. 9. An F pilus coated with phage MS2 virions and an uncoated F₀lac pilus from an adsorption mixture of *E. coli* CR34-1(F⁺) and *E. coli* JC6256(EDP208) with phage MS2 without formalin (sodium phosphotungstate negative stain, see text).

Fig. 10. A 711 pilus from *E. coli* 153-2(R711b) with MS2 phage virions adsorbed in the presence of formalin (uranyl acetate negative stain, see text).

Table 4. *Efficiency of adsorption and change in titre of phage MS2 when incubated with various strains of E. coli*

Strain	Approximate pili/cell*	MS2 adsorbed (%)†	Change in MS2 titre (%)‡
CR34-1(F ⁺)	1.0	74.1	+2200
CR34-2	0	5.3	-9
J53-2(R711b)	2.8	5.7	-16
JC6256(EDP208)	6.7	5.7	-19
CR34-2(EDP208)	3.6	5.3	-8

* Results from Bradley (1977), except for strains JC6256(EDP208) and CR34-2(EDP208), which were counted for the experiment under the same growth conditions.

† Difference between the surviving phage titre and that of a blank (2.28×10^6 p.f.u. ml⁻¹), expressed as a percentage of the blank.

‡ Difference between the titre of the cell-free culture fluids after 5 h incubation and that of a blank (broth with MS2 titre 2.1×10^4 p.f.u. ml⁻¹) incubated at the same time, expressed as a percentage of the blank.

Coexistence of F₀lac or EDP208 with R711b in E. coli strain CR34

Tests for incompatibility were carried out according to the criteria of Datta (see Coetzee *et al.*, 1972). When R711b was transferred to CR34-2 carrying F₀lac or EDP208, no loss of the Lac⁺ property was detected (45 transconjugants were tested in each case). Twenty clones each of the resulting doubles [CR34-2(R711b, EDP208) and CR34-2(R711b, F₀lac)] were tested for stability in overnight static broth cultures. No loss of the kanamycin resistance determined by R711b was detected and the reversion rate to Lac⁻ was one Lac⁻ colony or less for every 200 Lac⁺ for each clone tested when plated on MC plus kanamycin.

Ten clones of each double were tested for the separate transfer of the plasmids in matings with CR34-1. In every case, transfer of Lac⁺ without kanamycin resistance, and kanamycin resistance without Lac⁺ was observed, indicating that each plasmid was present and replicating autonomously. Because of the long-term instability of R711b, the stability of the doubles could only be measured in the short term. However, there was certainly no evidence of incompatibility. These results agree with those of N. Datta (personal communication).

DISCUSSION

The pili of F₀lac (Figs 5, 6), like those of R711b, resemble F pili, as previously mentioned (Brinton, 1965; Lawn *et al.*, 1967). The reason for their ability to confer sensitivity to filamentous but not RNA F-specific bacteriophages is not known. The results described here do not suggest that the pilus tips, which act as receptors for the filamentous phages, are serologically related. Thus it would appear more likely that the filamentous bacteriophages are non-specific in that they adsorb to the tips of different kinds of pili. Because F₀lac and 711 pili conferred the same phage sensitivity pattern, it seemed possible that they might be the same. However this is not the case, for each is serologically unrelated to the other as well as to other known F pilus serotypes. Adsorption of MS2 under various conditions has also revealed differences. EDP208 pili cannot be shown to adsorb the phage under any circumstances, whereas 711 pili show attached virions only after fixation, suggesting that a type of adsorption may occur that is reversible and, since no phage multiplication can be demonstrated, does not lead to infection.

The present classification of the X incompatibility group, to which R711b is assigned, is open to doubt (see Bradley, 1977). Therefore the possibility that R711b and F₀lac were related on the basis of incompatibility was considered. It is shown that they are not so related, this difference between them being reinforced by the differences in their pili.

It may be of evolutionary interest that the morphological form of the F pilus occurs with different properties and can be determined by unrelated plasmids. This morphology may

be the most efficient for the particular functional purposes of the pilus, and may have proliferated in nature by reason of some advantage, perhaps of a mechanical character.

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