Expression of Plasmids Coding for Colonization Factor Antigen II (CFA/II) and Enterotoxin Production in *Escherichia coli*

By P. MULLANY,¹ A. M. FIELD,² M. M. McCONNELL,^{1*}
S. M. SCOTLAND,¹ H. R. SMITH¹ AND B. ROWE¹

¹Division of Enteric Pathogens and ²Virus Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, U.K.

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Two plasmids transferred from enterotoxigenic *Escherichia coli* (ETEC) of serotype O6. H16 and biotypes A and C coded for mannose-resistant haemagglutination (MRHA) and production of heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT). Both plasmids were non-autotransferring being mobilized most efficiently by the R plasmid R100-1. They were similar in their genetic properties being incompatible with each other and plasmids of the Inc group FI. The wild-type strains produced the colonization factor antigen II (CFA/II) which was made up of different coli surface antigens (CS). The biotype A strains produced CS1 and CS3 while the biotype C strains produced CS2 and CS3. These three antigens have the ability to cause MRHA. When plasmids coding for MRHA were transferred to K12 strains, the degree of haemagglutination was markedly reduced and only CS3 was produced. When both plasmids were transferred back into biotype A strains, good MRHA was restored and the strains produced CS1 and CS3. In a biotype C strain CS2 and CS3 were formed. The production of the antigens was compared by enzyme-linked immunosorbent assay (ELISA). The strains were also examined by electron microscopy where it was found that CS1 and CS2 were fimbrial antigens while CS3 was not.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) can cause diarrhoeal disease in both humans and animals by colonizing the small intestine and producing enterotoxins. In many ETEC strains the adhesion to the intestinal mucosa has been found to be mediated by specific fimbrial antigens. Four antigens K88, K99, F41 and 987P have been identified on strains of animal origin (for review see Gaastra & de Graaf, 1982), while in ETEC isolated from humans the colonization factor antigens I and II (CFA/I and CFA/II) have been described (Evans *et al.*, 1975; Ørskov & Ørskov, 1977; Evans & Evans, 1978; Cravioto *et al.*, 1982; Smyth, 1982). The fimbrial antigen E8775 has also been demonstrated in some ETEC of human origin but its role in intestinal colonization has not yet been confirmed (Thomas *et al.*, 1982).

CFA/II was originally described as a heat-labile colonization factor antigen produced by enterotoxigenic *E. coli* of serogroups O6 and O8. It mediated mannose-resistant haemagglutination (MRHA) of bovine erythrocytes at 4 °C and was fimbrial in nature. When MRHA⁺ ETEC were examined by immunodiffusion tests using a specific CFA/II antiserum only a single precipitin line was observed (Evans & Evans, 1978). When other workers examined ETEC of serogroup O6 for the presence of CFA/II in a similar way, more than one line was detected (Cravioto *et al.*, 1982; Smyth, 1982).

Abbreviations: CFA, colonization factor antigen; CS, coli surface antigen; ETEC, enterotoxigenic *Escherichia coli*; LT, heat labile enterotoxin; MRHA, mannose-resistant haemagglutination; PT, PBS containing 0.02% Tween 20; PTF, PBS containing 0.01% Tween 20 and 0.5% foetal calf serum; ST, heat stable enterotoxin.

O6.H16 strains have been divided into seven types according to biochemical tests (Scotland *et al.*, 1977). Antisera were prepared against different biotypes of MRHA⁺ O6.H16 or O6.H⁻ strains and absorbed with the corresponding MRHA⁻ derivative. Using these antisera to test O6.H16 strains of different biotypes three distinct precipitin lines could be demonstrated. In most strains the antigen designated CFA/II component 3 (Cravioto *et al.*, 1982) or coli surface associated antigen 3 (CS3) (Smyth, 1982) was produced together with a second antigen, CFA/II component 1 or 2 (CS1 or CS2). A few strains made only CS2. CS1 production was always associated with O6.H16 biotype A strains while CS2 was produced by strains of biotypes B, C and F (Cravioto *et al.*, 1982; Smyth, 1982). Strains belonging to serogroups other than O6 were also examined and found only to produce CS3 (Cravioto *et al.*, 1982) with the exception of one O139.H28 strain which produced CS1 and CS3 (M. M. McConnell & S. M. Scotland, unpublished). In this paper, CFA/II is used as a general term to describe the three antigens CS1, CS2 and CS3. A CFA/II producing strain may express CS1 and CS3, CS2 and CS3 or CS2 or CS3 only. No wild-type strains producing only CS1 have been described. From an examination of immunodiffusion pictures and because a precipitin line was found in O8, O80 and O85 strains it is clear that CFA/II as defined by Evans & Evans (1978) corresponds to CS3 (component 3) (Cravioto *et al.*, 1982; Smyth, 1982).

Strains producing CFA/II have so far been found in the following serogroups: O6, O8, O78, O80, O85, O115, O139 and O168 (Evans & Evans, 1978; Cravioto *et al.*, 1982; Smyth, 1982; Thomas & Rowe, 1982). Some of these strains cause MRHA of human as well as bovine erythrocytes (Evans & Evans, 1978; Cravioto *et al.*, 1982; Smith *et al.*, 1983).

Production of CFA/II components has been shown to be plasmid mediated and linked to the production of heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) in strains of serogroups O6 and O85. A CFA/II-ST-LT plasmid was introduced into *E. coli* K12 from one of the O6.H16 strains by cotransformation with a drug-resistance plasmid; the K12 transformant carried a plasmid of molecular weight 60×10^6 (Penaranda *et al.*, 1980). CFA/II-ST-LT plasmids have also been mobilized from three O6.H16 strains belonging to biotype B or C which originally produced CS2 and CS3. After transfer of the plasmids to K12, only CS3 was expressed. However, when the same plasmid was transferred back into an O6.H16 biotype C strain both CS2 and CS3 were produced (Smith *et al.*, 1983).

In this paper we describe the transfer of a CFA/II plasmid from an O6.H16 strain of biotype A. The properties of this plasmid were compared with those of one of the plasmids isolated from an O6.H16 biotype C strain. Both plasmids were transferred from K12 into a number of different *E. coli* hosts and the expression of the CS antigens was examined by MRHA, immunodiffusion and enzyme-linked immunosorbent assay (ELISA). Some of the strains carrying CFA/II plasmids were examined by electron microscopy.

METHODS

Bacterial strains and plasmids. The wild-type ETEC strains producing CFA/II are listed in Table 1; these strains have been described previously (Cravioto *et al.*, 1982). Table 1 also shows non-enterotoxigenic strains used as recipients in experiments to study the expression of CFA/II.

The standard plasmids used in transfer and incompatibility experiments are listed in Table 2.

Tests for resistance to antibacterial drugs. Strains were tested for drug resistance by the methods of Anderson & Threlfall (1974).

Enterotoxin tests. ST was detected by the infant mouse test (Dean *et al.*, 1972). The production of LT was examined with Y1 adrenal cells (Donta *et al.*, 1974) and CHO cells (Guerrant *et al.*, 1974) in tissue culture.

Mannose-resistant and mannose-sensitive haemagglutination (MSHA). MRHA tests were performed as described by Cravioto *et al.* (1982). Blood samples from individual calves were supplied by Tissue Culture Services Ltd.

Strains to be tested for type 1 fimbriae which cause MSHA were grown statically in nutrient broth and were subcultured at least three times before being tested for their ability to agglutinate washed guinea pig erythrocytes at a concentration of 3% in saline with and without 0.5% mannose at room temperature (Duguid *et al.*, 1955).

Immunodiffusion tests (ID). Antisera were prepared in rabbits against PB-176 (*E. coli* O6.H16 biotype A which produces CS1 and CS3), E4833 (*E. coli* O6.H16 biotype B which produces CS2 and CS3) and E2047/69 (*E. coli* O6.H16 biotype B which produces only CS2). Antisera for CS1 and CS3, or CS2 and CS3 or CS2 only were obtained by absorption of whole sera with spontaneous CFA/II⁻ variants detected as MRHA⁻ variants of each

Table 1. *Properties of E. coli strains*

Strain no.	Origin	Serotype	Biotype	Enterotoxin production		Coli surface antigen		
				ST	LT	CS1	CS2	CS3
E9188	Japan, 1977	O6.H16	A	+	+	+	—	+
58R296	E9188	O6.H16	A	—	—	—	—	—
E8292	Gambia, 1977	O6.H16	A	+	+	+	—	+
58R665	E8292	O6.H16	A	—	—	—	—	—
E5470	Britain, 1976	O6.H16	C	+	+	—	+	+
E219/69	Sharjah, 1969	O6.H16	C	+	+	—	+	+
G176	E219/69*	O6.H16	C	—	—	—	—	—
G178	E3998*	O6.H1	NT	—	—	—	—	—
Properties								
54R310	O156.H ⁻ <i>fim</i> Nal ^r							
14R519	K12F ⁻ <i>lac</i> Nal ^r							
21R868	K12F ⁻ <i>lac</i> ⁺ Str ^r							

NT, Not tested.

* Nalidixic acid-resistant mutant.

Table 2. *Standard plasmids*

Plasmid*	Drug resistance markers†	Incompatibility group	Reference
R100-1	CmSmSuTc	FII	Jacob <i>et al.</i> (1977)
pMR5	ApKmTc	P	Robinson <i>et al.</i> (1980)
NTP2	SmSu	2	McConnell <i>et al.</i> (1979)
NTP107	Km	MP10	McConnell <i>et al.</i> (1979)

* NTP = non-autotransferring plasmid.

† Abbreviations for drug resistance markers: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Su, sulphathiazole; Tc, tetracycline.

strain. An antiserum to CS1 was prepared by using PB-176 as the vaccine strain and absorbing the resultant antiserum with E4833. An antiserum to CS3 was prepared by using E4833 as the vaccine strain and absorbing the resultant antiserum with E2047/69. The immunodiffusion tests were performed as described by Cravioto *et al.* (1982).

Transfer of plasmids from the wild-type strain. The wild-type strain E9188 was tested for the presence of transfer factors by attempting to mobilize the non-autotransferring resistance plasmids NTP2 and NTP107 (Anderson & Threlfall, 1974). Plasmids in E9188 were marked with the ampicillin resistance transposon Tn1 using the temperature-sensitive derivative of RP1 (pMR5) (Robinson *et al.*, 1980). The plasmids were then mobilized into K12 using the R factor R100-1.

Marking of plasmids in K12 by transposition and the formation of mutant plasmids. The kanamycin-resistance transposon Tn5 and the tetracycline-resistance transposon Tn10 were inserted into the CFA/II plasmid by the methods previously described (Smith *et al.*, 1982). A derivative of R100-1 which no longer coded for tetracycline resistance was used for mobilization of the CFA/II plasmids marked with Tc^r. Deletions in the CFA/II plasmid were produced by transposon loss (Smith *et al.*, 1982).

Phage tests and fertility inhibition. Broth cultures of K12 strains carrying a CFA/II plasmid and a derepressed F-like factor R100-1 were tested with phage μ 2 in surface spot tests. Plasmids that cause fertility inhibition (*fi*⁺) inhibit synthesis of F fimbriae by derepressed F-like factors and therefore reduce or abolish visible lysis of the culture by phage μ 2 (Anderson & Threlfall, 1974).

The ability of *E. coli* K12 carrying the CFA/II plasmids to restrict *E. coli* phages was tested by using surface spot tests as previously described (McConnell *et al.*, 1980).

Incompatibility experiments. The CFA/II plasmids were tested for their incompatibility relationships, i.e. their ability to co-exist stably with each other. NTP162 was mobilized by R100-1 into a K12 strain carrying NTP166 and the transconjugants tested for loss of drug resistance and MRHA (Table 3; Smith *et al.*, 1983).

Plasmid DNA studies. Plasmid DNA was partially purified from the wild-type strains and from K12 derivatives. For rapid screening the strains were lysed with alkaline sodium dodecyl sulphate as described by Birnboim & Doly

(1979). Molecular weights of the plasmids were determined by electrophoresis on vertical slab gels containing 0.6% (w/v) agarose (Sigma type II); plasmids of known molecular weight were run on the same gels.

Enzyme-linked immunosorbent assay (ELISA). (1) *Preparation of Immunoglobulin G (IgG).* Specific IgGs were precipitated with ammonium sulphate at 33% saturation. The mixture was allowed to equilibrate for 30 min at room temperature. After centrifugation at 20000 *g* at 4 °C for 20 min, the pellet was redissolved in a minimal volume of PBS (0.15 M-NaCl, 0.15 M-PO₄, pH 7.2). The IgG was then dialysed overnight at 4 °C against 0.01 M-sodium carbonate buffer (pH 9.5). The protein concentration was read at *A*₂₈₀.

(2) *Preparation of conjugate.* The IgG was labelled with horse-radish peroxidase as described by Smith & Tedder (1981).

(3) *Preparation of antigens.* The antigens were prepared by two methods, sonication and heat extraction. For sonication the cells were grown overnight on a 150 mm CFA agar plate (Evans & Evans, 1978), then resuspended in 10 ml PBS at an approximate concentration of 10¹⁰ cells ml⁻¹. The suspension was then sonicated for 7 min. The cell debris was removed by centrifugation at 30000 *g*. Samples (1 ml) were kept frozen at -30 °C. For heat extraction the cells from a CFA agar slope were resuspended in 1 ml saline and heated at 60 °C for 30 min.

(4) *Immunoassay.* Microtitre plates were coated with 200 µl specific IgG at a concentration of approximately 4 µg ml⁻¹ in carbonate buffer, pH 9.5, and left overnight at 4 °C. The plates were washed three times in PBS containing 0.02% (v/v) Tween 20 (PT). Antigen (100 µl) was added to each well. The antigens were prepared by heat extraction and used diluted 1:2, or prepared by sonication and used in doubling dilutions, in PBS containing 0.01% (v/v) Tween 20 and 0.5% (v/v) foetal calf serum (PTF). The plates were incubated for 2 h at 37 °C, and then washed three times in PT. Specific IgG conjugate (100 µl) diluted in PTF were added to each well on the plate. The optimum dilution of the conjugate was previously determined by titration against specific antigens. The plates were again incubated for 2 h at 37 °C and then washed as before. Substrate (100 µl) prepared by adding 9 mg *o*-phenylenediamine and 40 µl 30% hydrogen peroxide to 25 ml 0.1 M-phosphate buffer pH 6.0, was added to each well and the plates were incubated in the dark at room temperature for 1 h. Sulphuric acid (0.25 M; 25 µl) was added to each well to stop the reaction. The results were read on an ELISA reader (Titertek Multiskan, Dynatech) at 492 nm. The end point of the titration was taken as the dilution when the reading was five to ten times the level of the control strain.

Electron microscopy. The strains to be used for electron microscopy were grown on CFA agar for two successive 24 h periods to give maximum expression of CFA/II and suppress the production of type 1 fimbriae (Evans & Evans, 1978). These strains were examined for type 1 fimbriae by making a suspension of approximately 10¹¹ cells ml⁻¹ in saline and testing for MSHA. The cells were prepared for electron microscopy and immune electron microscopy according to the methods previously described (McConnell *et al.*, 1981).

RESULTS

Properties of the wild-type strains used as donors of CFA/II plasmids

The plasmids used in this study were transferred from two wild-type ETEC strains of serotype O6.H16, E9188 and E5470 (Table 2). Both these strains produced LT and ST, neither was drug-resistant or produced colicin. E9188 had previously been shown to belong to biotype A and produce CFA/II components CS1 and CS3, while E5470 belonged to biotype C and produced CFA/II components CS2 and CS3 (Cravioto *et al.*, 1982). The strains were initially identified by their ability to give MRHA of calf erythrocytes at 4 °C. E9188 also gave weak MRHA of human erythrocytes but E5470 did not. As with calf erythrocytes (Smith *et al.*, 1983), whether or not MRHA occurred depended on the individual from whom the erythrocytes were obtained. The other biotype A strain E8292 used in this study gave very good MRHA with human erythrocytes (Table 1). Strains of biotypes B, C and F had previously been shown not to give any haemagglutination of human erythrocytes (Cravioto *et al.*, 1982; Smith *et al.*, 1983).

E5470 had previously been shown to contain no transfer factors. Only one plasmid (NTP148) of a molecular weight of 59 × 10⁶ was identified by gel electrophoresis (Smith *et al.*, 1983). E9188 was tested for transfer factors as described in Methods, but no mobilization of NTP2 or NTP107 was observed. The strain contained five plasmids of molecular weights 59, 44, 6.1, 4.1 and 3.4 × 10⁶.

Transfer of a CFA/II plasmid

The single plasmid (NTP148) in the wild-type strain E5470 had previously been marked with the Ap^r transposon Tn1. The autotransferring F-like factor R100-1 was used to mobilize NTP148-Ap (NTP150) into the K12 strain 14R519 when it was found that insertion of Tn1 into

Table 3. Properties of CFA/II plasmids and their derivatives

Original strain	Plasmid no.	Drug resistance markers	Enterotoxin production		Coli surface antigen			10 ⁻⁶ × Molecular weight
			ST	LT	CS1*	CS2*	CS3	
E9188 O6.H16 biotype A	NTP155	—	+	+	+	—	+	59
	NTP166	Ap	+	+	+	—	+	62
	NTP167	ApTc	+	+	+	—	+	64
E5470 O6.H16 biotype C	NTP148	—	+	+	—	+	+	59
	NTP150	Ap	+	—	—	+	+	62
	NTP157	ApKm	+	—	—	+	+	64
	NTP161	ApKm	+	—	—	+	+	64
	NTP162	Km	—	—	—	—	—	17

* Property in MRHA-negative derivative of original strain.

NTP148 resulted in loss of LT production so that NTP150 coded for ST and MRHA (Smith *et al.*, 1983).

A similar method was used to try to transfer a plasmid coding for MRHA from E9188. Tn1 was used to mark plasmids in the wild-type strain as described in Methods. R100-1 was introduced into the wild-type strain and then used to mobilize Ap^r to 14R519. Ten out of ten Ap^r transconjugants caused MRHA and carried the R plasmid. In order to determine to which plasmid Ap^r was linked, a 30 min cross was performed using an Ap^r MRHA⁺ recombinant. The transconjugants were selected on MacConkey agar plates containing either ampicillin or chloramphenicol. Ten colonies from each selection were tested for Ap^r, Cm^r and MRHA.

From the cross eight transconjugants were obtained which were positive for all three markers. However five Cm^r colonies were found to contain only the R plasmid R100-1 and three Ap^r colonies were negative for Cm^r showing that Ap^r was not linked to R100-1. Four Ap^rCm^r MRHA⁺ colonies were identified showing that a plasmid which did not confer MRHA was marked with Ap^r. A strain carrying one of these plasmids was tested for enterotoxins and found to be negative. All the MRHA⁺ transconjugants (eight Ap^rCm^r MRHA⁺, two Ap^rMRHA⁺) were Ap^r so that it was also possible that the MRHA plasmid was marked with this resistance.

Further short crosses were performed from two Ap^rCm^rMRHA⁺ transconjugants again selecting separately for Ap^r and Cm^r colonies. From twenty Cm^r transconjugants one was obtained which also gave MRHA but was negative for Ap^r, showing that these two properties could be separated. Two Ap^r MRHA⁺ colonies were obtained without the R plasmid. From one of these, Ap^r was lost, but the transconjugant was still MRHA⁺.

The Ap^r MRHA⁺ transconjugant carried two plasmids of molecular weights 48 and 59 × 10⁶. After loss of Ap^r, the plasmid of molecular weight 59 × 10⁶ (NTP155) was left. Toxin and immunodiffusion tests showed that the K12 strain carrying this plasmid produced LT, ST and CS3, but no CS1 was detected. However, transfer of the marked derivative of NTP155, NTP167 (see next section and Table 3) into a MRHA negative derivative of E9188 (58R296) showed that the plasmid still coded for CS1 as well as CS3, since this strain produced both antigens.

Marking of NTP148 and NTP155 by transposition and isolation of mutants in K12

NTP148 had been marked with Tn1 in the wild-type strain to give NTP150 with loss of the ability to produce LT (Table 3, Smith *et al.*, 1983). NTP150 was subsequently marked with Tn5 as described in Methods, in two independent experiments, to give CFA/II plasmids coding for Ap, Km and ST, NTP157 and NTP161. A deletion mutant, NTP162, was isolated by loss of Ap^r from NTP161 with loss of the ability to produce CS antigens, and ST.

In order to compare the properties of the plasmids from two different biotypes and to examine the expression of the CS antigens in different host strains, NTP155 was marked with Ap^r (NTP166) and Ap^r Tc^r (NTP167) by transposition as described in Methods (Table 3).

Table 4. *Expression of CS antigens in different host strains*

Strain	Test	Plasmid no.:	
		NTP167	NTP157
O6.H16	MRHA Calf RBC	++++	++++
biotype A	Human RBC	+	+
58R296	ID and ELISA	CS1, CS3	CS1, CS3
O6.H16	MRHA Calf RBC	++++	++++
biotype A	Human RBC	++++	++++
58R665	ID and ELISA	CS1, CS3	CS1, CS3
O6.H16	MRHA Calf RBC	+++	+++
biotype C	Human RBC	—	—
G176	ID and ELISA	CS2, CS3	CS2, CS3
O6.H1	MRHA Calf RBC	+	—
G178	ID and ELISA	CS3	CS3
O156.H ⁻ <i>fim</i>	MRHA Calf RBC	++	+
54R310	ID and ELISA	CS3	CS3
<i>E. coli</i> K12	MRHA Calf RBC	++	+
21R868	Human RBC	—	—
	ID and ELISA	CS3	CS3

RBC, Red blood cells.

+ to + + + +, Degree of haemagglutination; —, no haemagglutination.

Properties of these plasmids

These are listed in Table 3. NTP150 and NTP166 were mainly used to examine the genetic properties of the two plasmids. Strains carrying both plasmids gave fertility inhibition of $\mu 2$ phage but did not restrict any of the range of phages described in Methods. Both were non-autotransferring and were mobilized by F-like R plasmids. R100-1 mobilized NTP150 and NTP166 into K12 in an 18 h cross at a frequency of 10^{-1} to 10^{-2} per recipient cell, but with other F-like R plasmids the frequency of transfer was lower (Smith *et al.*, 1983). Incompatibility tests showed that NTP162, a CS1-CS3-ST⁻ derivative of NTP150, and NTP166 were incompatible with each other and belonged to Inc group FI.

Expression of plasmids in different host strains

Previous work had shown that K12, O8.H9, O25.H42 and O78.H⁻ strains containing NTP150 only expressed one CFA/II component, CS3 (Smith *et al.*, 1983). NTP167 and NTP157 were mobilized into a number of different recipient strains. Transconjugants which did not contain the R factor were examined for MRHA of calf and human erythrocytes and by immunodiffusion and ELISA. The results are shown in Table 4.

When NTP157 and NTP167 were transferred to O156.H⁻, O6.H1 or K12 strains, only one CFA/II component, CS3, was expressed. In O6.H16 strains, CS3 was also expressed along with another antigen CS1 or CS2. Which of these latter antigens was produced depended on the biotype of the host; biotype A strains expressed CS1 and biotype C strains CS2 in addition to CS3. NTP157 was also transferred to O6.H16 strains of biotype B and F when CS2 and CS3 were expressed (results not shown).

Strains which only produced CS3 gave a very weak haemagglutination with calf erythrocytes and none with human erythrocytes. The strains producing CS1 or CS2 as well as CS3 gave much better haemagglutination than CS3 only strains. The CS1⁺, CS3⁺ strains gave the best haemagglutination. CS1⁺, CS3⁺ strains also caused haemagglutination of human erythrocytes, although the degree of haemagglutination varied with the host strain, while CS2⁺ CS3⁺ strains did not cause any haemagglutination of these erythrocytes (Table 4).

Titration of CS antigen production

The amount of the CS antigens produced by different strains carrying the two plasmids NTP167 and NTP157 when they were present in different host strains was compared using the

Table 5. Production of CS antigens as measured by ELISA

Host strain	Plasmid	Production of antigen*		
		CS1	CS2	CS3
O6.H16 biotype A 58R296	NTP167	2 ¹⁰	NAD	2 ¹⁰
	NTP157	2 ¹¹	NAD	2 ⁵
O6.H16 biotype C G176	NTP167	NAD	2 ¹¹	2 ¹⁵
	NTP157	NAD	2 ¹¹	2 ¹¹
O156.H ⁻ <i>fim</i> 54R310	NTP167	NAD	NAD	2 ¹¹
	NTP157	NAD	NAD	2 ¹¹
<i>E. coli</i> K12 21R868	NTP167	NAD	NAD	2 ¹⁶
	NTP157	NAD	NAD	2 ¹¹

NAD, No antigen detected.

* The CS titre was expressed as the highest dilution which gave a reading five to ten times that of the control.

Table 6. Electron microscopy of strains containing CFA/II plasmids

Strain	Plasmid	Antigens produced	No. of cells counted	No. of cells with degree of fimbriation*			Percentage fimbriate
				Many	Moderate	Few	
O6.H16 biotype A 58R296	None	None	212	0	0	0	0
	NTP167	CS1, CS3	175	89	38	25	87
	NTP157	CS1, CS3	109	91	0	1	84
O6.H16 biotype C G176	None	None	246	0	2	4	2.5
	NTP167	CS2, CS3	147	73	45	18	92
	NTP157	CS2, CS3	115	70	45	0	100
O156.H ⁻ <i>fim</i> 54R310	None	None	608	6	1	2	1.5
	NTP167	CS3	167	2	0	2	2.5
	NTP157	CS3	480	0	0	2	0.5
<i>E. coli</i> K12 21R868	None	None	173	NT	NT	NT	9
	NTP166	CS3	130	2	0	0	1.5
	NTP150	CS3	104	2	0	2	3.8

NT, Not tested.

* Many, >200 fimbriae per cell; moderate, 20–200 fimbriae per cell; few, <20 fimbriae per cell.

ELISA (Table 5). This assay was only semi-quantitative but was very sensitive for the detection of the antigens; CS1 and CS2 could be detected at a dilution up to 2¹⁵. The tests for CS1 and CS2 with extracts of O156.H⁻ or K12 strains carrying the plasmids were completely negative, while CS3 production occurred at a similar level or higher than that in the O6 strains. Strains carrying NTP167 usually produced more CS3 than the corresponding strain carrying NTP157. The experiments also showed that there was no apparent cross-reaction between any of the antigens.

Electron microscopy

For these experiments strains carrying the plasmids marked with two resistances NTP167 and NTP157 were used except for K12 strains which carried the plasmids with one drug-resistance marker NTP166 and NTP150. Examination of negatively-stained preparations of cultures by electron microscopy showed that strains without plasmids coding for any CFA/II antigens were mainly non-fimbriate (Table 6). Strains producing CS1 or CS2 consisted mainly of fimbriate cells, while cultures which only produced CS3 were mainly non-fimbriate like the original host strain. The few fimbriae seen on cells devoid of CS1 and CS2 antigens were

Fig. 1. Fimbriae from G176 with NTP157, expressing CS2 and CS3 antigens, treated with CS2 antiserum. The fimbriae are heavily coated with antibody. Negative stain $\times 150000$.

Fig. 2. Fimbriae from G176 with NTP157, expressing CS2 and CS3 antigens, treated with CS1 antiserum. The fimbriae are not coated with antibody. Negative stain $\times 150000$.

Fig. 3. 58R296 with NTP167, expressing CS1 and CS3 antigens, treated with CS1 antiserum. All the fimbriae attached to the bacterium and some of the loose fimbriae are heavily coated with antibody; other loose fimbriae are not coated. Negative stain $\times 48000$.

Fig. 4. Fimbriae from 58R296 with NTP167, expressing CS1 and CS3 antigens, treated with CS1 antiserum. Some loose fimbriae are heavily coated with antibody and others are not coated. Negative stain $\times 150000$.

Table 7. Immune electron microscopy

Strain	Serum	Plasmid no.	
		NTP167	NTP157
O6.H16	Anti-CS1	+++/—	+++/—
biotype A	Anti-CS2	—	—
58R296	Anti-CS3	—	—
O6.H16	Anti-CS1	—	—
biotype C	Anti-CS2	+++	+++
G176	Anti-CS3	—	—
<i>E. coli</i> K12	Anti-CS1	NT	NT
21R868	Anti-CS2	NT	—
	Anti-CS3	NT	—

NT, Not tested.

+++ , fimbriae heavily coated with antibody; — , fimbriae not coated with antibody; ++ +/— , attached fimbriae and some loose fimbriae heavily coated with antibody, some loose fimbriae not coated with antibody.

probably type 1 fimbriae which have previously been seen on cells grown in these culture conditions (McConnell *et al.*, 1981). The antigenic nature of the fimbriae was investigated in some strains by immune electron microscopy using CS1, CS2 and CS3 antisera to coat the fimbriae on the cells. It was seen that CS1 and CS2 antibodies attached only to fimbriae of cells producing the homologous antigens (Figs 1 and 2; Table 7). Immune electron microscopy failed to identify any binding site for CS3 antibody on the cell body, on the few fimbriae observed on cells producing only CS3, or on the fimbriae produced by strains exhibiting CS1 and CS3 or CS2 and CS3. The CS1 and CS2 fimbriae were morphologically indistinguishable and measured approximately 6.5 nm in width. The CS2 antibody coated all attached and loose fimbriae in cultures of cells producing CS2, but it was found that CS1 antibody failed to coat some of the loose fimbriae in cultures of cells producing CS1 while coating all attached fimbriae (Figs 3 and 4).

DISCUSSION

The experiments described show that the plasmids coding for CFA/II, ST and LT found in *E. coli* O6.H16 strains of different biotypes are very similar. The important factor determining which CFA/II component, CS1 or CS2, is produced is the genetic character of the host strain which is recognized by its biotype. The biotype of the O6.H16 strain is probably not important *per se* in determining which CS antigen is produced, since strains of biotypes B, C and F all produce CS2, but the chromosomal genes which influence whether CS1 or CS2 are produced have not been identified. When the same plasmids are transferred to strains of other serotypes such as O6.H1, O156.H[–], O8.H9 and *E. coli* K12, only CS3 is expressed. The only other serotype we have found which expresses CS1 as well as CS3 is a single O139.H28 strain isolated in Egypt. Another strain of this serotype produces CS3 only and not CS1 or CS2 when carrying a CFA/II plasmid (M. M. McConnell & S. M. Scotland, unpublished).

Since transfer of a CFA/II plasmid to strains of serotypes other than O6.H16 gives strains which produce only CS3, we have been able to look at the properties of the CS3 antigen separately and compare these with those of strains carrying both antigens. Electron microscopy shows that CS3 is non-fimbrial; therefore the fimbriae seen on CS1⁺ CS3⁺ or CS2⁺ CS3⁺ strains are the CS1 and CS2 antigens. The haemagglutination obtained with CS3-producing strains and calf blood was always very poor. With erythrocytes from some calves no haemagglutination was seen. This might explain why Smyth (1982) did not consider CS3 to be a haemagglutinin. Strains producing CS1 gave good haemagglutination of calf erythrocytes. Strains producing CS2 gave different degrees of haemagglutination from very good to none at all with erythrocytes from different calves (unpublished work; Faris *et al.*, 1982). Several reports

have said that CFA/II strains do not haemagglutinate human erythrocytes (Ørskov & Ørskov, 1977; Evans & Evans, 1978; Smyth, 1982), while we have found that strains producing CS1 frequently do, once again the result depends on the particular human erythrocytes used (unpublished work; Cravioto *et al.*, 1982).

In a survey of *E. coli* of different serotypes and from a variety of sources it was found that many strains possessed surface haemagglutinins which could bind erythrocytes. The ability of strains to cause MRHA or MSHA was usually associated with the presence of fimbriae. However, a few strains were found which caused MRHA but did not possess fimbriae (Duguid *et al.*, 1979; Ip *et al.*, 1981). The adhesion and ability to cause MRHA of some porcine ETEC was also mediated by non-fimbriate material (Awad-Masalmeh *et al.*, 1982). A mannose-specific lectin has been isolated from an *E. coli* strain causing a urinary tract infection which agglutinates yeast cells and human epithelial cells and differs from the fimbrial type 1 protein (Eshdat *et al.*, 1978). The O6 strains we have examined cause MRHA by fimbrial and non-fimbrial antigens, while the CFA/II strains of other serogroups only form the non-fimbrial antigen.

We have not been able to detect any cross-reaction between CS1 and CS2 by ELISA or immune electron microscopy. It may be that two structural genes are present on CFA/II plasmids which are switched on separately in different hosts, or one structural gene may be present and the gene product may be modified differently in the two hosts to produce antigenically distinct fimbriae. DNA and amino-acid sequencing should help to distinguish these two possibilities.

The presence of loose fimbriae to which no antibody was attached when they were treated with CS1 antiserum suggested that these O6 strains were producing a small number of fimbriae which differed antigenically from the much larger number of CS1 fimbriae formed. The strain might be producing type 1 fimbriae, although none were observed when the control strain was examined. The nature of these fimbriae is being investigated.

The results of antigen testing obtained by immunodiffusion were confirmed by ELISA. However, this test has been shown to be more sensitive than immunodiffusion. Strains carrying one mutant plasmid NTP156 were originally found to be negative for CS3 by immunodiffusion tests. ELISA titrations showed that some CS3 was coded for by this plasmid, although it was less than the wild-type strain (results not shown).

An O8.H9 strain which only produces CS3 has been shown to attach to human intestinal epithelial cells (Wadstrom *et al.*, 1980) suggesting that CS3 is an adhesin (Smyth, 1982). O6.H16 strains produce this antigen as well as the CS1 or CS2 fimbriae so that it is not clear if the fimbriae are required for adherence. The role of these fimbriae in pathogenicity requires testing using strains which do not produce CS3.

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