Characterization of a Putative Colonization Factor (PCFO166) of Enterotoxigenic *Escherichia coli* of Serogroup O166

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Enterotoxigenic *Escherichia coli* (ETEC) of serogroup O166 gave mannose-resistant haemagglutination (MRHA) with bovine and human erythrocytes. The strains did not react with antisera prepared against the known colonization factors CFA/I, CFA/II, CFA/III, CFA/IV and PCFO159:H4. Strain E7476 of serotype O166:H27, which produced heat-stable enterotoxin (ST), was examined initially. It produced fimbriae about 7 nm in diameter. On SDS-PAGE two possible fimbrial polypeptides of molecular mass 15.5 and 17.0 kDa were seen. When variants of strain E7476 were isolated, loss of ST and MRHA together was associated with loss of a 98 MDa plasmid, while loss of ST alone correlated with plasmid deletion. An absorbed anti-strain E7476 antiserum reacted specifically with the 15.5 and 17.0 kDa polypeptides in Western immunoblotting and bound to the intact fimbriae by immuno-electron microscopy. When this antiserum was used in an ELISA to examine other strains of serogroup O166, a positive reaction was obtained with all the ST- and MRHA-positive strains. One strain of serotype O71:H27 and two strains of serotype O98:H⁻ also reacted with the absorbed anti-strain E7476 antiserum. The antiserum did not react with ETEC carrying known colonization factors. *E. coli* K12 and a number of *E. coli* of different serotypes carrying a plasmid coding for ST transferred from strain E7476, all gave MRHA and reacted with the absorbed anti-strain E7476 antiserum. The term putative colonization factor O166 (PCFO166) is proposed to describe the adhesive factor(s) on ETEC of serogroup O166 because of the similarity of properties with those of known colonization factors.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are a leading cause of diarrhoea in infants in developing countries and in travellers to these countries (Sack, 1975; Merson *et al.*, 1980). The ability of ETEC to adhere to, and colonize the small intestine is essential for pathogenicity and is due to colonization factors. These are usually fimbriae, many of which have the ability to agglutinate red blood cells from a number of animal species in the presence of mannose (Parry & Rooke, 1985; De Graaf & Mooi, 1986). A number of adhesins have now been identified in ETEC of human origin, viz. colonization factor antigen (CFA)/I, CFA/II, CFA/III and CFA/IV (PCF8775) and PCFO159:H4 (Evans *et al.*, 1975; Evans & Evans, 1978; Honda *et al.*, 1984; Thomas *et al.*, 1985; Tacket *et al.*, 1987; McConnell *et al.*, 1988). CFA/I, CFA/III and PCFO159:H4 are single fimbrial antigens while CFA/II and CFA/IV are both antigen complexes. Strains producing CFA/II possess one or other of the fimbrial antigens CS1 or CS2 and a fibrillar antigen CS3, or may possess CS3 alone (Cravioto *et al.*, 1982; Smyth, 1982); ETEC producing CFA/IV have the fimbrial antigen CS4 or CS5 and a second antigen CS6

Abbreviations: CFA, colonization factor antigen; CS, coli surface antigen; ETEC, enterotoxigenic *Escherichia coli*; LT, heat-labile enterotoxin; MRHA, mannose-resistant haemagglutination; PCF, putative colonization factor; ST, heat-stable enterotoxin.

(Thomas *et al.*, 1985). ETEC producing CS5-like fimbriae or only CS6 have also been identified (McConnell *et al.*, 1986; McConnell & Rowe, 1989). Other possible colonization factors have also been described, viz. INT407 on ETEC of serotype O27:H7 or O27:H20 (Thomas, 1985), colonization factors on ETEC of serotype O148:H28 (Knutton *et al.*, 1987; McConnell *et al.*, 1988) and 2230 on a strain of serotype O25:H16 (Darfeuille-Michaud *et al.*, 1986).

Surveys of ETEC have shown that each colonization factor is associated with particular O serogroups and enterotoxin types. Some appear to be very specific. CS1 with CS3 has only been reported in strains of serotype O6:H16 or H⁻ which produce heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) and one ST⁺LT⁺ strain of serotype O139:H28 (Cravioto *et al.*, 1982; Smyth, 1982; Scotland *et al.*, 1985); CS2 with CS3 in ST⁺LT⁺ strains of serotype O6:H16 or H⁻ (Cravioto *et al.*, 1982; Smyth, 1982); CFA/III with CS6 in LT⁺ strains of serotype O25:H16 or H⁻ (Honda *et al.*, 1983, 1984; McConnell *et al.*, 1988; McConnell & Rowe, 1989); CS4 with CS6, in ST⁺LT⁺ strains of serotype O25:H42 (Thomas *et al.*, 1985; McConnell *et al.*, 1985) and PCFO159:H4 in ST⁺LT⁺ strains of serotype O159:H4 or H20 (Tacket *et al.*, 1987; McConnell & Rowe, 1989).

The range of O serogroups of strains carrying other factors is much more diverse. CFA/I has been reported in ST⁺ and ST⁺LT⁺ strains of serogroups O4, O7, O15, O20, O25, O63, O78, O90, O104, O110, O126, O128, O136, O153 and O159 (Evans & Evans, 1978; Cravioto *et al.*, 1982; Thomas & Rowe, 1982; Levine *et al.*, 1983; Changchawalit *et al.*, 1984; Darfeuille-Michaud *et al.*, 1987); CS3 as a single adhesive factor in ST⁺LT⁺ strains of serogroups O8, O78, O80, O85, O115, O128, O139 and O168 (Evans & Evans, 1978; Cravioto *et al.*, 1982; Thomas & Rowe, 1982; Levine *et al.*, 1983; Scotland *et al.*, 1985); CS5 together with CS6 in ST⁺ strains of O6, O29, O92, O114, O115 and O167 (Thomas *et al.*, 1985; McConnell *et al.*, 1985; Manning *et al.*, 1987; McConnell & Rowe, 1989); CS5-like fimbriae in LT⁺ST⁺ strains of serotype O15 and LT⁺ strains of serotype O114, (McConnell & Rowe, 1989) and CS6 in ST⁺ strains of serogroups O27, O92, O148, O153, O159 and O169 (McConnell *et al.*, 1985, 1986). However, the same surveys also show that there are still some serogroups of ETEC where no colonization factor has been identified. These ETEC frequently produce only LT (Changchawalit *et al.*, 1984; McConnell *et al.*, 1985; Cravioto *et al.*, 1988; McConnell & Rowe, 1989). Since it has been suggested that ETEC vaccines could be prepared using adhesins, it is important to try to identify the colonization factors on these strains.

In the Division of Enteric Pathogens (DEP) there is a large collection of ETEC which have been serotyped. These strains come principally from Asia, but also included are strains from South America, Africa, the USA and Europe. Approximately 1200 strains received between 1975 and 1986 were confirmed as enterotoxigenic. Among these strains about 17% belong to 25 serogroups where no colonization factor has been identified. Of these strains approximately 30% produce ST, 60% produce LT and 10% produce ST and LT. Strains of serogroup O166 formed the largest group that produce ST only. It was decided to look for a possible colonization factor in these strains and then to examine other ETEC for this factor.

METHODS

Bacterial strains. Seventeen ST-producing and one ST-negative *E. coli* of serogroup O166, including strain E7476 (Scotland *et al.*, 1979), were examined (Table 1). These strains were all negative for LT. Most of these strains have been described previously (Gross *et al.*, 1985). Since enterotoxin production is coded for by plasmids which may be lost from strains on storage (Evans & Evans, 1978), a single colony of each culture was used which was shown to be positive for ST production immediately before the start of these tests, e.g. strain E7476A (Table 1).

Ninety-nine ETEC with known colonization factors, i.e. CFA/I, CFA/II, CFA/III, CFA/IV or PCFO159:H4, and 106 ETEC with no known colonization factor, were tested. Included in the 106 ETEC with no known colonization factor were LT⁺ strains of serogroups O8, O15 and O159, since colonization factors had only been found in ST⁺ or ST⁺LT⁺ strains of these serogroups, and strains of serogroups O7 and O20, since, although CFA/I has been reported in strains of these serogroups it has not been detected in strains of these two serogroups characterized by the DEP.

Non-enterotoxigenic nalidixic-acid-resistant strains of serotypes O6:H16, O40:H3, O71:H48, O128:H10, O128:H49, O154:H4 and O159:H34, into which the autotransferring ST plasmid TP224::Tn10 from strain

Table 1. *ETEC* of serogroup O166

Strain*	Country of isolation	No. of similar strains	ST production	MSHA (Guinea-pig)	MRHA		ELISA†	Drug resistance‡	Plasmid content (MDa)§
					Bovine	Human			
E7476A	South Africa	0	+	—	+	+	+	sens	98
E7476B		0	—	—	—	—	—	sens	—
E7476C		0	—	—	+	+	+	sens	88
E5540		0	+	—	+	+	+	Sm Su	98 , 4.9
E10680	Bangladesh	0	+	+	+	+	+	Tc	88 , 55
E10728		1	+	—	+	+	+	Tc	100 , 39.5
E14514		3	+	—	+	+	+	sens	98
E17063		1	+	—	+	+	+	sens	90 , 54
E17738		0	+	—	+	+	+	Tc	84 , 50, 4.8
E17751		3	+	—	+	+	+	Tc	88 , 55
E18521		0	—	—	—	—	—	Tc	50
E33679	Yugoslavia	0	+	+	+	+	+	Ap	62 , 20.5, 5.3, 4.5

* The strains were all of flagellar type H27 except strain E33679 which was H48.

† ELISA against absorbed anti-strain E7476 antiserum.

‡ Symbols for drug resistance: Ap, ampicillin; Sm, streptomycin; Su, sulphonamides; Tc, tetracycline; sens, sensitive.

§ Plasmids in **bold** type code for ST.

E7476 had been transferred (Scotland *et al.*, 1979) were also tested. These strains have been described previously (Scotland *et al.*, 1983).

Tests for resistance to antibacterial drugs and colicin E2. Strains were tested for drug resistance by the methods of Anderson & Threlfall (1974). Colicin resistance was detected by the agar overlay method of Frédéricq (1957).

Enterotoxin tests for ST and LT. Two methods of testing were used for each enterotoxin. For small numbers of strains ST production was detected in the infant mouse (Dean *et al.*, 1972) and LT production with Y1 adrenal cells in tissue culture as previously described (Donta *et al.*, 1974). For testing a large number of single colonies, DNA probes for ST and LT were used. These comprised synthetic oligonucleotides labelled with alkaline phosphatase and supplied in kit form (SNAP^R system; DuPont-NEN). The ST probe contained both STA1- and STA2-specific sequences. Hybridization to colonies was done by methods recommended by the manufacturer (McConnell *et al.*, 1988).

Mannose-sensitive and mannose-resistant haemagglutination (MSHA and MRHA). For testing for type-1 fimbriae, strains were grown statically in nutrient broth (Difco). Samples (1 ml) of culture were centrifuged and the cells resuspended in 0.2 ml of nutrient broth for testing for MSHA with guinea-pig red blood cells as previously described (Cravioto *et al.*, 1982). The strains were subcultured every 48 h for up to 12 times for MSHA testing.

For the first tests from solid medium the strains were grown on MacConkey agar and then overnight on CFA agar and tested for MSHA and MRHA of bovine, human and guinea-pig red blood cells as previously described (Cravioto *et al.*, 1982). Freshly drawn Rhesus-positive group O human blood was used. For most tests the strains were grown overnight on CFA agar containing Bacto-Bile salts no. 3 (Difco) (1.5 g l⁻¹).

Isolation of MRHA-negative and ST-negative variants of strain E7476. Three strategies were tried: (a) one hundred colonies were picked from MacConkey plates to CFA slopes and tested for MRHA. (b) Approximately 500 colonies were replicated onto filters and tested for ST genes with a DNA probe. (c) Plasmid TP224:Tn10 was transferred into strain E7476A in an overnight cross. Tetracycline-resistant transconjugants were selected on MacConkey agar plates containing tetracycline (10 µg ml⁻¹) and colicin E2 (10³ units ml⁻¹) to counter-select against the donor. One transconjugant was grown overnight in nutrient broth, plated onto nutrient agar and then replicated onto nutrient agar plates containing tetracycline (10 µg ml⁻¹). Colonies sensitive to tetracycline were examined further.

Preparation of antiserum. An antiserum was raised against strain E7476A by injecting rabbits intraperitoneally with a formalinized suspension containing about 10⁹ bacteria ml⁻¹ as previously described (Thomas *et al.*, 1985). The antiserum was absorbed with an MRHA-negative derivative, variant E7476B (Table 1).

Immunodiffusion. The antisera were tested by the Ouchterlony gel immunodiffusion technique as previously described (Cravioto *et al.*, 1982). Antigens were prepared by suspending cultures from a 150 mm diameter plate containing CFA agar with bile salts (1.5 g l⁻¹) in 1.5 ml saline (0.15 M) and heating the suspensions for 30 min at 60 °C. The slides were examined for precipitin lines for 24 to 48 h. In some cases the slides were washed in saline for 48 h followed by a wash in distilled water for a further 24 h. Then they were dried and stained at 37 °C for 30 min with Coomassie blue (Weeke, 1973).

Plasmid DNA studies. Plasmid DNA was prepared by the method of Birnboim & Doly (1979) and separated by electrophoresis on 0.6% (w/v) agarose gels as described previously (Willshaw *et al.*, 1979). Molecular masses of the plasmids were determined relative to plasmid standards run on the same gel.

DNA hybridization. Plasmids encoding production of ST were identified with a specific ST probe. Plasmid DNAs of wild-type strains and ST-negative variants were separated by agarose gel electrophoresis and blotted to nylon membranes (Hybond N; Amersham). Gels were acid treated (0.25 M-HCl) before denaturation of the DNA. The DNA was bound to the filter by baking (80 °C, 2 h). The ST probe was as described in the enterotoxin test section. Hybridization to blotted plasmid DNA was by methods recommended by the manufacturer.

Preparation of fimbriae. Crude preparations of fimbriae were prepared by suspending bacteria from 150 mm CFA agar plates containing bile salts in 1.5 ml saline (0.15 M) and heating the suspensions for 30 min at 60 °C. They were centrifuged in a microcentrifuge (5000 g, 5 min) and the supernatant fractions harvested. Following protein determinations (Lowry method) preparations were stored at -10 °C.

SDS-PAGE. This was done by the method of Laemmli (1970) using vertical slab gels. Extracts were run on a 20% (w/v) polyacrylamide gel for 3.5 h at 50 mA. Proteins of known molecular mass were run on the same gel. Immunoblotting was performed as described by Stevenson & Griffiths (1985). Protein profiles immobilized on nitrocellulose sheets were reacted with the absorbed anti-strain E7476A antiserum and antibody-antigen complexes detected using an anti-rabbit IgG antibody (Miles Scientific) radiolabelled with ¹²⁵I. Each profile was reacted with approximately 5 µg IgG containing 10⁶ c.p.m.

Electron microscopy. The strains to be used for electron microscopy were grown on MacConkey agar then on CFA agar for 24 h to give maximum expression of the colonization factor(s) and to suppress the production of type-1 fimbriae. The strains were tested for type-1 fimbriae production by checking for MSHA of guinea-pig red blood cells. The bacteria were prepared for electron microscopy and immuno-electron microscopy as previously described (McConnell *et al.*, 1981).

ELISA. Strains to be tested in the ELISA were first streaked on MacConkey agar before overnight incubation on CFA agar slopes. The cultures were checked for MRHA and suspended in 2 ml 0.05 M-carbonate/bicarbonate buffer (pH 9.6) and heated at 60 °C for 30 min. The ELISAs were done as previously described (McConnell & Rowe, 1989). The heated suspensions were used to coat the microtitre plates. CFAs on the ETEC were identified with specific absorbed antisera or IgG. Binding of the antibody was detected using anti-rabbit IgG peroxidase conjugate. The substrate was prepared by adding 9 mg *o*-phenylenediamine and 40 µl 30% (v/v) H₂O₂ to 25 ml 0.1 M-sodium phosphate buffer (pH 6.0); 100 µl was added to each well and the plates were incubated in the dark at room temperature for 10 min. Sulphuric acid (2 M, 25 µl) was added to each well to stop the reaction.

RESULTS

Properties of strain E7476

Strain E7476 was examined by Scotland *et al.* (1979) as part of a study of enterotoxin plasmids of drug-resistant strains of *E. coli* of human origin. It was reported at that time as belonging to an unidentifiable O serogroup, possessing flagellar antigen H27, producing ST but not LT, being resistant to streptomycin and sulphonamides and possessing two plasmids. The O serogroup to which strain E7476 belongs has now been designated O166 (Gross *et al.*, 1985). A single ST-positive colony of strain E7476 (designated E7476A) was selected for examination for a colonization factor. It was no longer drug resistant and contained only one plasmid (Table 1).

When strain E7476 was originally tested after transfer from Dorset egg medium to CFA agar and overnight incubation, no MRHA of human, guinea-pig or bovine red blood cells was detected (Gross *et al.*, 1985). However, when strain E7476A was streaked on MacConkey agar and then onto CFA agar, strong MRHA of human and bovine red blood cells occurred, while MRHA of guinea-pig red blood cells was still negative. This technique had previously been shown to improve the expression of MRHA with a strain of serotype O15:H11 (Thomas, 1985). When strain E7476A was streaked directly from a Dorset egg slope to MacConkey, Nutrient, Brain Heart Infusion or Mueller-Hinton agar no MRHA was detected. It was subsequently found that if strain E7476A was transferred directly from a Dorset egg slope to CFA agar containing bile salts, the culture gave good MRHA when mixed with bovine or human red blood cells. Strain E7476A was negative for MSHA of guinea-pig blood after growth in nutrient broth indicating the absence of type-1 fimbriae.

Strain E7476A was tested in ELISAs with antisera against the colonization factors CFA/I, CFA/II (CS1, CS2, CS3), CFA/III, CFA/IV (CS4, CS5, CS6) and PCFO159:H4. These sera were found not to react with strain E7476A.

Fig. 1. Immuno-electron microscopy of fimbriate strain E7476A. (a) Strain E7476A showing fimbriae of about 7 nm diameter. (b) Fimbriate strain E7476A treated with absorbed antiserum raised against E7476A. Antibody molecules are attached to the fimbriae. Negative stain, 1% phosphotungstic acid (pH 6.4). Bar, 100 nm.

Isolation of MRHA- and ST-negative variants of strain E7476

One hundred colonies, picked from a MacConkey agar plate to CFA agar slopes, were tested for MRHA with bovine red blood cells. All were MRHA positive.

Since many colonization factors are coded for by the same plasmid as codes for enterotoxin production (McConnell *et al.*, 1981; Smith *et al.*, 1983; Thomas *et al.*, 1987) it was decided to look for an ST-negative variant of strain E7476 which might also be MRHA-negative. About 500 colonies were replicated onto filters and tested with the ST probe. One ST-negative colony (variant E7476C) was isolated. It was still MRHA-positive and contained an 88 MDa plasmid (Table 1), indicating a deletion to the 98 MDa plasmid of strain E7476A.

A third attempt to isolate a MRHA-ST-negative colony was made. Plasmid TP224::Tn10 was transferred into strain E7476A; the transconjugants were still MRHA positive. One transconjugant was tested for loss of tetracycline resistance. In total, 1500 colonies of this transconjugant were examined and two tetracycline-sensitive colonies were isolated. One colony (variant E7476B) was tested and found to be ST⁻MRHA⁻ and contained no plasmid DNA (Table 1).

Characterization of strain E7476A and variants E7476B and E7476C

An antiserum was prepared to strain E7476A and absorbed twice with variant E7476B. Heated suspensions of strains E7476A, B and C were tested by immunodiffusion against absorbed anti-strain E7476A antiserum. A single immunoprecipitate was seen between the antigen and antiserum wells with suspensions of strains E7476A and C. No immunoprecipitate was observed with variant E7476B.

Before electron microscopy the strains were grown on solid media. No type-1 fimbriae were detected by MSHA of guinea-pig red blood cells. Fimbriae of about 7 nm diameter were produced by strain E7476A (Fig. 1a) and variant E7476C. No fimbriae were detected on variant E7476B. The absorbed anti-strain E7476A antiserum bound to fimbriae of strain E7476A *in situ* (Fig. 1b) and variant E7476C (result not shown).

Fig. 2. SDS-PAGE profiles of fimbrial preparations from MRHA-positive and -negative variants of strain E7476. (a) Coomassie-blue-stained profiles; (b) autoradiogram of a Western immunoblot showing extracts reacted with antiserum raised against strain E7476A which had been absorbed with variant E7476B. Protein profiles were reacted with 30 μ l antiserum followed by 5 μ g ^{125} I-labelled anti-rabbit antibody (10^6 c.p.m.). Strain E7476A (lanes 1 and 4); variant E7476B (lanes 2 and 5); variant E7476C (lanes 3 and 6). Strains E7476A and E7476C, which are both MRHA positive and fimbriate, expressed two polypeptides of 15.5 and 17.0 kDa (lanes 1 and 3), which were absent from the MRHA-negative derivative E7476B. These two polypeptides reacted with the absorbed antiserum (lanes 4 and 6) raised against strain E7476A as above. The protein load was 30 μ g per lane.

When fimbrial preparations of strains E7476A and C were run on SDS-PAGE two prominent polypeptide bands of molecular masses approximately 15.5 and 17.0 kDa were observed (Fig. 2a). No prominent bands of this size were seen on the E7476B track. These polypeptides reacted with the absorbed anti-strain E7476A antiserum (Fig. 2b).

Screening of other strains of serogroup O166

Seventeen strains of serogroup O166 from the DEP culture collection were tested. Sixteen of the strains produced ST and were MRHA positive with bovine and human red blood cells (Table 1). Fifteen of these strains possessed the flagellar antigen H27 and one, strain E33679, the flagellar antigen H48. The ST-positive strains gave a positive reaction in an ELISA with antiserum raised against strain E7476A (Table 1). Five MRHA positive strains – E5540, E14514, E17063, E17738 and E17751 – were examined by electron microscopy and were seen to be fimbriate. All 17 strains were negative in ELISAs when tested with antisera raised to CFA/I, CFA/II, CFA/III, CFA/IV and PCFO159:H4.

All the ST-positive strains except strain E33679 contained a plasmid of molecular mass 84 to 100 MDa, i.e. similar in size to plasmid TP224 (Scotland *et al.* 1979). These plasmids all hybridized with the ST probe (Table 1). Strain E33679 had four plasmids, the largest of which (molecular mass 62 MDa), hybridized with the ST probe (Table 1). When heat-released extracts of MRHA-positive strains were examined by SDS-PAGE, all 16 strains were found to produce polypeptides of 15.5 and 17.0 kDa which reacted with the absorbed anti-strain E7476A antiserum in immunoblots.

One strain of serotype O166:H27, E18521, had been sent to the DEP as ST positive but was negative on testing. Strain E18521 did not give MRHA of bovine or human red blood cells and

Table 2. Reaction of ETEC with known and no known colonization factors with absorbed anti-strain E7476A antiserum

Colonization factor	Enterotoxin	Total no. of strains	Serogroup*
CFA/I	ST or ST and LT	21	O4(3) O63(4) O78(4) O126(5) O128(3) O136(1) O153(1)
CFA/II	ST and LT	13	O6(4) O8(3) O85(2) O115(2) O139(2)
CFA/III + CS6	LT	5	O25(5)
CFA/IV	ST or LT or ST and LT	56	O15(3) O25(4) O27(5) O29(1) O79(1) O85(1) O114(22) O115(3) O148(4) O153(2) O159(4) O167(4) O169(2)
PCFO159:H4	ST and LT	4	O159(4)
None of above	ST	14	O2(1) O9(1) O20(3) O30(1) O34(3) O49(1) O64(2) O71(1)† O76(1)
None of above	LT	76	O7(11) O8(2) O9(1) O15(2) O17(6) O20(1) O21(2) O40(1) O41(1) O48(1) O59(1) O64(3) O79(1) O80(1) O88(8) O101(2) O104(1) O112(2) O114(10) O117(5) O146(2) O159(12)
None of above	ST and LT	16	O1(1) O7(1) O17(1) O20(6) O60(1) O98(2)† O109(3) O159(1)
		205	

* The numbers of strains of each serogroup tested are given in brackets.

† These strains reacted with absorbed anti-strain E7476A antiserum

gave a negative reaction in the ELISA with the absorbed anti-strain E7476A antiserum. It appeared to have lost the plasmid which codes for ST and MRHA (Table 1).

Testing of ETEC of other serotypes by ELISA

Ninety-nine strains producing known colonization factors were tested with absorbed anti-strain E7476A antiserum in an ELISA using heated suspensions and all were found to be negative (Table 2).

One hundred-and-six ETEC which did not possess any known colonization factors were also tested with the absorbed E7476A antiserum (Table 2). Three strains reacted with the antiserum, two ST⁺LT⁺ strains of serotype O98:H⁻ and one ST⁺ strain of serotype O71:H27. Like the strains of serogroup O166, they all gave MRHA with bovine and human red blood cells, contained a plasmid of 84 to 100 MDa and produced two prominent polypeptides of 15.5 and 17.0 kDa which reacted with the absorbed anti-strain E7476A antiserum in immunoblots.

Examination of *E. coli* strains carrying plasmid TP224::Tn10

When an *E. coli* K12 strain and *E. coli* strains of serotypes O6:H16, O40:H3, O71:H48, O128:H10, O128:H49, O154:H4 and O159:H34 containing plasmid TP224::Tn10 were streaked onto CFA agar containing bile salts and tested for MRHA of bovine red blood cells, all were positive although the reaction of the *E. coli* K12 strain was weak. When heated suspensions were tested in the ELISA with the absorbed anti-strain E7476 antiserum all were positive.

DISCUSSION

The ability of pathogenic *E. coli* of both human and animal origin to agglutinate red blood cells in the presence of mannose frequently correlates with the possession of colonization factors (Parry & Rooke, 1985; De Graaf & Mooi, 1986). When strains belonging to serogroup O166 were initially tested they did not cause MRHA of bovine, human or guinea-pig red blood cells (Gross *et al.*, 1985). However, expression of MRHA is dependent on the medium on which a strain is cultured (Parry & Rooke, 1985). Thomas (1985) observed that a strain of serotype O15:H11 only gave good MRHA if it was grown on MacConkey agar before incubation on CFA agar. When the strains of serogroup O166 were cultured in this way, the ST-positive strains all agglutinated bovine and human red blood cells in the presence of mannose. It was

subsequently found that if one of the ingredients of MacConkey agar, bile salts, was added to the CFA agar the initial incubation step on MacConkey agar could be omitted.

The fimbriae produced by the type strain E7476 were rigid rod-shaped structures, similar to those of CFA/I, CS1, CS2, CS4, CFA/III and PCFO159:H4 but antigenically distinct. The inability of the variant E7476B to give MRHA was associated with the absence of fimbriae on the bacterial cells. Variant E7476B did not produce ST and had lost a 98 MDa plasmid. When this 98 MDa plasmid (TP224) marked with tetracycline resistance to give plasmid TP224::Tn10 was transferred to *E. coli* K12 or other serotypes of *E. coli* the transconjugants possessed MRHA ability and produced ST. Therefore plasmid TP224 coded for enterotoxin and fimbriae production like other plasmids which coded for known colonization factors and enterotoxins, e.g. CFA/I-ST, CFA/II-ST-LT, CS5-CS6-ST and PCFO159:H4-ST-LT (McConnell *et al.*, 1981, 1988; Smith *et al.*, 1983; Tacket *et al.*, 1987).

The similarity of the properties of E7476 fimbriae to those of other colonization factors and the ability of fimbriate E7476 cells to adhere to human enterocytes (S. Knutton, personal communication) suggest that these fimbriae are also a colonization factor. The adhesin has provisionally been designated putative colonization factor O166 (PCFO166). It was also detected in other strains of serogroup O166, two strains of serotype O98:H⁻ and one of serotype O71:H27. Although PCFO166 was found in only a few serogroups of *E. coli* in this survey, the fact that it was coded for by an autotransferring plasmid and could be expressed in *E. coli* of all the serogroups tested means that in time it may become more widely dispersed.

When fimbrial preparations of strain E7476 and other strains of the same serogroup were analysed by SDS-PAGE, two polypeptides of typical fimbrial subunit size were seen in MRHA-positive but not in MRHA-negative strains. Although both polypeptides reacted with the absorbed antiserum prepared against strain E7476A, only one immunoprecipitate was seen on immunodiffusion, suggesting that the fimbriae may be composed of two subunits of different molecular mass rather than that two distinct types of fimbriae with a different subunit structure were present. However, we have previously found that immunodiffusion tests are not entirely reliable (McConnell *et al.*, 1985) so that further work is necessary to establish the exact nature of the surface protein(s) and to examine more closely their role in adhesion.

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