Properties of Wild-type Strains of Enterotoxigenic Escherichia coli Which Produce Colonization Factor Antigen II, and Belong to Serogroups Other Than O6

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Enterotoxigenic strains of Escherichia coli, which belonged to serogroups other than O6 and produced colonization factor antigen II, usually produced only coli surface antigen 3 (CS3) and gave weak mannose-resistant haemagglutination of bovine erythrocytes. A non-autotransferring plasmid, NTP165, from a strain of E. coli 0168.H16 coded for heat-stable enterotoxin, heat-labile enterotoxin and CS antigens. The CS antigens expressed after acquisition of plasmid NTP165 depended on the recipient strain: a biotype A strain of serotype O6. H16 expressed CS1 and CS3; a biotype C strain of serotype O6. H16 expressed CS2 and CS3; strain K12 and strain E19446 of serotype O139. H28 expressed only CS3. An exceptional wild-type strain, E24377, of serotype O139. H28 produced CS1 and CS3 when isolated; a variant of E24377 which had lost the plasmid coding for CS antigens produced both CS1 and CS3 after the introduction of NTP165.

INTRODUCTION

Specific surface antigens produced by some enterotoxigenic strains of Escherichia coli (ETEC) are considered to be responsible for adhesion to the intestinal mucosa of man and animals (for reviews see Gaastra & de Graaf, 1982; Levine et al., 1983a). In most cases these antigens are associated with the ability to cause mannose-resistant haemagglutination (MRHA) of erythrocytes of certain species. The surface antigen, termed colonization factor antigen II (CFA/II), was described in ETEC from human sources (Evans & Evans, 1978). CFA/II has been found in ETEC belonging to serogroups O6, O8, O9, O78, O80, O85, O115, O128 and O139 (Evans & Evans, 1978; Cravioto et al., 1982; Thomas & Rowe, 1982; Levine et al., 1983b; Peñaranda et al., 1983). Immunodiffusion studies have shown that CFA/II is made up of more than one antigenic component or coli surface antigen (CS) (Cravioto et al., 1982; Smyth, 1982). In general, ETEC of serogroup O6 belonging to biotype A (as defined by Scotland et al., 1977) produce CS1 and CS3; ETEC of serogroup O6 belonging to other biotypes produce CS2 and CS3; strains of serogroups other than O6 produce only CS3. In the present communication CFA/II is used as a general term to describe any of the three antigens CS1, CS2 and CS3.

Most CFA/II-positive ETEC produce both the heat-stable enterotoxin (ST) and the heat-labile toxin (LT). Genetic studies of strains of E. coli O6 have shown that genes for production of ST, LT and CFA/II are carried on a single plasmid (Peñaranda et al., 1980; Smith et al., 1983; Mullany et al., 1983). The present paper describes ETEC strains producing CFA/II which
belong to serogroups other than O6. A plasmid coding for CS antigens was transferred from one strain so that the properties of this plasmid could be studied and compared to those of plasmids isolated from the \textit{E. coli} O6 strains.

\section*{METHODS}

\textit{Bacterial strains.} Strains of ETEC were studied which produced CFA/II in immunodiffusion studies but which did not belong to serotype O6. H16. Representative strains were chosen of every serogroup available in the culture collection of the Division of Enteric Pathogens. Also included were strains of the same serogroup but from different geographical areas. The strains are listed in Table 1. Many had been identified in a previous survey of CFA production in ETEC (Cravioto \textit{et al.}, 1982) in which CFA/II+ strains belonging to serogroups O6, O8, O80, O85 and O115 were reported.

The strains were characterized with respect to production of ST and LT, drug resistance and colicin production by methods described previously (Scotland \textit{et al.}, 1979).

\textit{Immunodiffusion tests.} These were done as described previously (Cravioto \textit{et al.}, 1982; Mullany \textit{et al.}, 1983). Antisera were prepared against strain PB-176 (O6. H16 CS1+ CS3+), strain E4833 (O6. H16 CS2+ CS3+) and strain E7463 (O8. H9 CS3+); these sera were absorbed with spontaneous CFA/II variants (detected as MRHA- variants) of each strain, resulting in antisera giving a precipitin reaction with CS1 and CS3, CS2 and CS3, or CS3 alone (Cravioto \textit{et al.}, 1982). To prepare antigens, bacteria were grown at 37 °C for 18 h on a large (15 cm) plate of CFA agar (Evans \textit{et al.}, 1977): the growth was then suspended in saline (0.85\%, 1-5 ml), heated at 60 °C for 20 min, and used without dilution.

\textit{Enzyme-linked immunosorbent assay (ELISA).} Antigens CS1, CS2 and CS3 were detected by an ELISA as described previously (Mullany \textit{et al.}, 1983). To prepare the antigen single colonies were grown on CFA agar slopes overnight. All the growth was suspended in 1 ml saline and the suspension was heated at 60 °C for 30 min.

\textit{Haemagglutination tests.} MRHA of bovine erythrocytes was done in the cold as described previously (Cravioto \textit{et al.}, 1982) using bacteria grown on CFA agar. Blood samples from individual animals were supplied by Tissue Culture Services and the Cattle Blood Typing Service, Animal Breeding Research Organization, Edinburgh, UK. To test the ability to produce type 1 fimbriae, bacteria were grown in nutrient broth (Difco; 10 ml) without shaking for 24 h at 37 °C. The culture was centrifuged and the deposit resuspended in 1 ml saline. The bacterial suspension was tested for haemagglutination of guinea-pig erythrocytes in the presence and absence of D-mannose (0.5\%, w/v) at room temperature (Duguid \textit{et al.}, 1955). Strains were subcultured at least 15 times before it was concluded that type 1 fimbriae were not produced.

\textit{MRHA-negative variants of wild-type strains.} Cultures of wild-type strains kept at room temperature on Dorset egg slopes (Cowan & Steel, 1974) were plated out and 100 individual colonies were tested for the ability to give MRHA. When no spontaneous MRHA- variants were found an attempt was made to displace a plasmid coding for MRHA by incompatibility using plasmid NTP162 (see ‘Characterization of plasmids’ section).

The MRHA- variants obtained spontaneously or by incompatibility experiments were examined for production of ST, LT and CS antigens, and by electron microscopy.

\textit{Electron microscopy (EM).} Strains grown on CFA agar were examined by EM after negative staining with phosphotungstic acid (1\%, w/v, pH 6-4) (McConnell \textit{et al.}, 1981). The strains were subcultured daily on CFA agar slopes until at least two successive tests for mannose-sensitive haemagglutination (MSHA) of guinea-pig erythrocytes were negative in order that the production of type 1 fimbriae should be reduced as much as possible. All strains failed to give MSHA by five subcultures. It was confirmed that these suspensions still gave MRHA of bovine erythrocytes. The fimbriation of at least 75 bacteria was determined by EM.

\textit{Transfer of plasmids.} Transfer of antibiotic resistance plasmids was tested directly from wild-type strains to \textit{E. coli} K12, strain 14R519, which was nalidixic acid-resistant and lactose non-fermenting. Wild-type strains were tested for the presence of transfer factors by attempting to mobilize the non-autotransferring resistance plasmids NTP2, coding for resistance to streptomycin and sulphonamide (SmSu), or NTP107, coding for resistance to kanamycin (Km), into strain 14R519 (Anderson & Threlfall, 1974). Mobilization of non-autotransferring plasmids present in wild-type strains was attempted using the derepressed F-like resistance plasmid R100-1 (Jacob \textit{et al.}, 1977) which mobilizes CFA/II plasmids from strains of \textit{E. coli} O6 (Smith \textit{et al.}, 1983; Mullany \textit{et al.}, 1983). In addition to strain 14R519, another K12 strain, 21R868, was used as a recipient; this strain was streptomycin-resistant and lactose-fermenting. Two strains of \textit{E. coli} O6. H16 were also used as recipients: S8R296 (biotype A) and G176 (biotype C) (Mullany \textit{et al.}, 1983).

\textit{Characterization of plasmids.} The plasmid content of wild-type strains and K12 transconjugants was determined by agarose gel electrophoresis (Willshaw \textit{et al.}, 1979) of DNA prepared by the method of Birnboim & Doly (1979). Plasmids were marked with drug resistance by methods described previously (Smith \textit{et al.}, 1983). Tests for fertility inhibition and incompatibility were those of Mullany \textit{et al.} (1983). For incompatibility tests plasmid NTP162 was used. A plasmid, NTP148, coding for ST, LT and CS antigens in strain E5470 (\textit{E. coli} O6. H16) had been marked...
Table 1. *Origin and properties of wild-type* E. coli *strains*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Origin</th>
<th>Serotype</th>
<th>Enterotoxin production</th>
<th>CFA/II components</th>
<th>Type 1 fimbriae production†</th>
<th>10⁻⁶ × Mol. wt of plasmid‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ST LT MRHA* CS1 CS2 CS3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E8794</td>
<td>Bangladesh</td>
<td>O8.H9</td>
<td>+ + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E9034</td>
<td>Mexico</td>
<td>O8.H9</td>
<td>+ + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E11939</td>
<td>South Africa</td>
<td>O8.H9</td>
<td>+ + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E11175</td>
<td>Bangladesh</td>
<td>O78.H12</td>
<td>+ + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E6670</td>
<td>Brazil</td>
<td>O80.H9</td>
<td>+ + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E5264</td>
<td>Vietnam</td>
<td>O85.H7</td>
<td>+ + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E9136</td>
<td>Mexico</td>
<td>O85.H7</td>
<td>+ + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E8783</td>
<td>Bangladesh</td>
<td>O115.H51</td>
<td>+ + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E19446</td>
<td>Thailand</td>
<td>O139.H28</td>
<td>+ + + + + + + + + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E24377</td>
<td>Egypt</td>
<td>O139.H28</td>
<td>+ + + + + + + + + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E10710</td>
<td>Bangladesh</td>
<td>O168.H16</td>
<td>+ + + + + + + + + + - - - +</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The degree of mannose-resistant haemagglutination of bovine erythrocytes depended on the animal used (see text), but the most common result is shown: +, weak reaction; ++ +, strong reaction.
† Detected by mannose-sensitive haemagglutination of guinea-pig erythrocytes.
‡ Plasmids of the sizes in bold type are those missing from MRHA⁻ variants; variants were obtained spontaneously except those from E19446 and E24377 which were from incompatibility experiments.

with ampicillin resistance (Ap) and then kanamycin resistance (Km). NTP162 was derived from this plasmid by deletion of DNA coding for Ap, ST, LT and CS (Smith et al., 1983). NTP162 was introduced into wild-type strains by mobilization with plasmid R100-1 (Jacob et al., 1977). Variants which did not carry R100-1 were selected for further tests.

RESULTS

Properties of wild-type strains

The serotypes and countries of origin of the wild-type strains are given in Table 1. All strains produced ST and LT.

With the exception of strain E24377, the strains gave weak to moderate MRHA of erythrocytes from some calves but no haemagglutination of erythrocytes from others. In one survey, erythrocytes from 30 calves which had been characterized with respect to over 60 blood sub-types were compared; MRHA was moderate with three, weak with 23 and undetected with four. No correlation was demonstrated between MRHA reaction and the blood types of the animals studied. Strain E24377 gave strong MRHA of bovine erythrocytes and was shown to produce CS1 and CS3 by immunodiffusion tests. All other strains in this study produced CS3 only.

All strains except E24377 gave MSHA of guinea-pig erythrocytes before the sixth successive culture in broth, indicating the production of type 1 fimbriae. Strain E24377 gave no MSHA even after 15 successive subcultures.

None of the strains produced colicins. Only two strains were resistant to antibacterial agents; strain E6670 was resistant to sulphomamide and tetracycline (Tc), and strain E24377 to streptomycin and sulphonamide.

Properties of MRHA-negative variants of wild-type strains

MRHA⁻ variants were isolated from strains E9034, E11939, E6670, E10710, E19446 and E24377. In all the variants, the loss of the ability to give MRHA of bovine erythrocytes was accompanied by the loss of the ability to produce CS3 (and CS1 in the case of E24377). The variants also failed to produce ST or LT. They also lacked a plasmid which was present in the wild-type strain although the molecular weight of these plasmids ranged from about 50 × 10⁶ in the two E. coli O8 strains to 85 × 10⁶ in the strains of E. coli O168 (Table 1).
Examination of strains by EM

Strains E9034, E11939, E6670, E9136, E8783, E19446, E24377 and E10710 were examined for the presence of fimbrial or fibrillar appendages or any unusual surface structures. Of the bacteria of strain E24377 94% were fimbriate with more than 200 fimbriae, which resembled those previously seen on organisms with colonization factor II (Mullany et al., 1983). Less than 20% of the bacteria of the other seven strains were fimbriate and usually fewer than 20 fimbriae, of this same morphological type, were seen on each bacterium. For three strains, E9034, E9136 and E10710, less than 1% of the bacteria were fimbriate. In all the EM preparations shapeless patches of finely granular material were noted; this material was seen free in the background and was also seen closely associated with some of the bacteria. When MRHA− CS− variants of six strains (E9034, E11939, E6670, E19446, E24377 and E10710) were examined by EM none of this extracellular material was seen.

Transfer of a plasmid coding for CS3

Plasmid analysis of the MRHA− CS− variants of certain wild-type strains in the present work indicated the presence of plasmids coding for CS, ST and LT. An attempt was made to transfer a plasmid coding for CS3 from the wild-type strains to the E. coli K12 derivative 14R519. Transconjugants were tested for the presence of CS3 by MRHA or by ELISA.

Resistance to tetracycline was transferred directly from strain E6670 but none of 10 Tc′ transconjugants of strain 14R519 were CS3+. There was no transfer of streptomycin resistance from strain E24377 to strain 14R519. All of the strains were tested for transfer factors, except strain E6670 which had already been shown to transfer a drug resistance plasmid; no other transfer factors were found.

The resistance plasmid R100-l was introduced into strains E9034, E11939, E6670, E9136, E8783, E24377 and E10710 in an attempt to mobilize any CS3 plasmid which might be present. From each mating experiment 20 K12 transconjugants, selected as carrying R100-l, were examined for co-transfer of CS3. All transconjugants were negative for CS3 except in the experiment with strain E10710 (R100-l) when 9 of 20 transconjugants were CS3+ in the ELISA. One CS3+ transconjugant, 58R595, was examined and shown to produce LT and ST; it carried two plasmids of molecular weight 64 × 10^6 and 85 × 10^6. When R100-l was lost from 58R595 one plasmid (NTP165) with a molecular weight of 85 × 10^6 remained; NTP165 coded for LT, ST and CS3 in K12.

Properties of NTP165

Plasmid NTP165 was fi+ like other CFA/I plasmids (Smith et al., 1983; Mullany et al., 1983). NTP165 was marked with Ap′ as described in Methods to give NTP165-Ap which still encoded CS, LT and ST in K12; it was also marked with Tc′ to give NTP165-Tc which coded for CS3 and LT but not ST.

The expression of CFA/I plasmids transferred from strains of E. coli O6. H16 had been tested in strain 58R296 (E. coli O6. H16, biotype A) and strain G176 (E. coli O6. H16, biotype C) by Mullany et al. (1983). These recipient strains were derived from enterotoxigenic, CFA/I+ wild-type strains but each had lost the plasmid coding for these properties. NTP165-Tc was mobilized by plasmid R100-l into strains 58R296 and G176 to examine the expression of CS antigens. Tetracycline-resistant transconjugants from both recipients gave strong MRHA of bovine erythrocytes. Immunodiffusion tests and the ELISA showed that strain 58R296 carrying NTP165-Tc produced CS1 and CS3 whilst strain G176 carrying NTP165-Tc produced CS2 and CS3 (Table 2). NTP165-Tc was also transferred into the MRHA− variants of the two strains of E. coli O139. H28. E19446 carrying NTP165-Tc expressed only CS3 and gave weak MRHA; E24377 carrying NTP165-Tc expressed both CS1 and CS3, and gave strong MRHA (Table 2). Also included in Table 2 are results of ELISA with a K12 strain, 21R868, carrying NTP165-Tc.

NTP165-Ap was tested for incompatibility with the non-autotransferring plasmid NTP162, derived from strain E5470 (E. coli O6. H16, biotype C). NTP162 was mobilized by plasmid R100-l into a K12 strain carrying NTP165-Ap; all 10 Km′ transconjugants tested had lost ampicillin resistance, indicating that the two plasmids were incompatible.
Table 2. Expression of CS antigens by strains carrying plasmid NTP165-Tc measured by ELISA

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Serotype</th>
<th>Plasmid</th>
<th>CS1</th>
<th>CS2</th>
<th>CS3</th>
<th>Absorbance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>58R296</td>
<td>O6. H16 (biotype A)</td>
<td>+</td>
<td>1.871</td>
<td>0.052</td>
<td>1.104</td>
<td></td>
</tr>
<tr>
<td>G176</td>
<td>O6. H16 (biotype C)</td>
<td>-</td>
<td>0.181</td>
<td>0</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>E19466†</td>
<td>O139. H28</td>
<td>+</td>
<td>0.140</td>
<td>1.572</td>
<td>1.095</td>
<td></td>
</tr>
<tr>
<td>E24377†</td>
<td>O139. H28</td>
<td>-</td>
<td>0.018</td>
<td>0.193</td>
<td>0.119</td>
<td></td>
</tr>
<tr>
<td>21R868</td>
<td>O rough H48</td>
<td>+</td>
<td>0.185</td>
<td>0.045</td>
<td>0.925</td>
<td></td>
</tr>
</tbody>
</table>

* Absorbance was read at 490 nm using specific IgG conjugates against CS1, CS2 and CS3. The test strains were measured against blanks prepared in the same way except that the antigen was replaced by saline.
† MRHA− derivatives were used which had lost the plasmid coding for CS antigens and toxin production.

Incompatibility of NTP162 in wild-type strains

NTP162 was incompatible with NTP165-Ap coding for CS, LT and ST in a strain of E. coli O168. H16. CS plasmids were not transferred from the other wild-type strains in this study so NTP162 was introduced into them to see if such a plasmid would be displaced. NTP162 was mobilized by R100-l into the wild-type strains E9034, E11939, E6670, E9136, E8783, E19446 and E24377. A total of 10 Km† transconjugants from each cross was tested for MRHA; all had lost the ability to give MRHA except for the cross with E19446 in which 9 of the 10 were MRHA−. Three to five MRHA− transconjugants from each mating were tested for CS3 and all were CS3−. These transconjugants were also ST− LT−.

DISCUSSION

Most ETEC of serogroup O6 which possess CFA/II produce CS1 and CS3, or CS2 and CS3 (Cravioto et al., 1982; Smyth, 1982). In contrast, the majority of CFA/II+ strains of other serogroups produced only CS3. An exception was strain E24377 (E. coli O139. H28) which produced CS1 in addition to CS3 and this is the only strain amongst over 100 CFA/II+ strains belonging to serogroups other than O6 in the culture collection of the Division of Enteric Pathogens.

The degree of MRHA of bovine erythrocytes by CS3+ strains depended greatly on the animal from which the erythrocytes were obtained, and we were unable to correlate this with the blood type of the animal. Similar variation of MRHA with the source of bovine erythrocytes has been reported for CFA/II+ strains of E. coli O168 (Faris et al., 1982; Mullany et al., 1983). In the present study CS3+ strains gave no MRHA with erythrocytes from some calves and this may explain the report of CS3+ MRHA− strains of E. coli O8 by Smyth (1984).

A plasmid, NTP165, coding for CS3, ST and LT was transferred from strain E10710. Incompatibility studies and examination of MRHA− variants demonstrated the presence of a plasmid coding for similar properties in seven other strains, although the molecular weights of such plasmids ranged from $52 \times 10^6$ to $85 \times 10^6$. Loss of CFA/II, ST and LT together from strains not belonging to serogroup O6 was reported by Evans & Evans (1978) and Peñaranda et al. (1980, 1983). In the latter study plasmids absent from the CFA/II− strains ranged in molecular weight from $50 \times 10^6$ to $78 \times 10^6$.

The expression of plasmid NTP165 derived from a CS3+ strain of E. coli O168. H16 resembled that of CFA/II plasmids derived from a CS1+ CS3+ strain of E. coli O6. H16 biotype A and from a CS2+ CS3+ strain of E. coli O6. H16 biotype C (Smith et al., 1983; Mullany et al., 1983). Acquisition of any of the three plasmids resulted in expression of CS3 only in K12, CS1
and CS3 in *E. coli* O6. H16 biotype A and CS2 and CS3 in *E. coli* O6. H16 biotype C. Thus all three plasmids encoded CS1, CS2 and CS3 but a property of the recipient determined which CS antigens were expressed. The expression of CS antigens was different in two strains of serotype O139. H28. One strain, E19446, only produced CS3 when isolated or after the introduction of NTP165 into a CS3- variant; the second, E24377, produced CS1 in addition to CS3 when isolated or after the introduction of NTP165 into a CS3- variant. Strain E24377 lacked the ability to produce type 1 fimbriae but it seems unlikely that this is directly related to the ability to produce CS1 because strains of *E. coli* O6. H16 which produced type 1 fimbriae and those which did not were both able to produce CS1 and CS3 (unpublished results).

In previous EM studies of CFA/II+ strains of *E. coli* O6. H16 and of transconjugants which had acquired a CFA/II plasmid from them (Mullany et al., 1983; Smyth, 1984) it was concluded that CS1 and CS2 were fimbrial, whereas CS3 was not. Examination by EM of the wild-type strains belonging to serogroup other than O6 in the present study supported the conclusion that CS3 is not fimbrial, and Smyth (1984) has also reported that a CS3+ strain of *E. coli* O8. H9 lacked fimbriae. Although we were unable to show an organized structure of CS3 by EM, extracellular granular material was present in all preparations of CS3+ strains examined and absent in preparations of MRHA- (CS3-) variants. However, in a study which included strains E9034 and E24377 and used EM aided by immune EM, Levine et al. (1984) reported the visualization of CS3 as fine, 2 nm wide fibrils on the bacterial surface projecting only one-third of the distance that the 6 nm wide, CS1 fimbriae projected from the surface. The phosphotungstic acid stain we used differed from the ammonium molybdate used by Levine and colleagues, which may account for the different morphology.

Many of the studies of CFA/II+ strains using animal models were done before the heterogeneity of CFA/II was recognized, and it is not possible to ascribe the results specifically to either CS1 (or CS2) or CS3 (Evans & Evans, 1978; Deetz et al., 1979; Wadström et al., 1980; Boedeker et al., 1982). In the prevention of ETEC disease, oral immunization using vaccines containing adhesive factors is likely to be important, and future studies involving CFA/II+ strains should use strains which have been characterized for their CS antigens.

**REFERENCES**


Non-O6 enterotoxigenic E. coli with CFA/II


