INFECTION AND IMMUNITY, Mar. 1982, p. 1119-1124
0019-9567/82/031119-06$02.00/0

Vol. 35, No. 3

New Fimbrial Antigenic Type (E8775) That May Represent a Colonization Factor in Enterotoxigenic *Escherichia coli* in Humans

LINDA V. THOMAS, ALEJANDRO CRAVIOTO,† SYLVIA M. SCOTLAND, AND BERNARD ROWE*

Division of Enteric Pathogens, Central Public Health Laboratory, London NW9 5HT, England

Received 8 September 1981/Accepted 6 November 1981

An enterotoxigenic strain of *Escherichia coli* O25:H42 (strain E8775), isolated from a patient in Bangladesh with diarrhea, caused mannose-resistant hemagglutination (MRHA) of human and bovine erythrocytes. The strain did not show slide agglutination or immunodiffusion precipitin lines with antiserum specific for the colonization factor antigen CFA/I or CFA/II. A variant *E. coli* strain, E8775-B, did not cause MRHA or produce enterotoxin. Electron microscopy revealed the presence of fimbriae on the surface of strain E8775 but not strain E8775-B. When strain E8775 was grown at 22°C, it became MRHA negative and fimbriae were absent. An antiserum prepared against strain E8775 was absorbed with strain E8775-B to make an antiserum specific for the fimbrial antigen. Using this absorbed antiserum, we found the fimbrial antigen in 48 of 742 enterotoxigenic *E. coli* strains. The 48 strains belonged to serogroups O25, O115, and O167. It is suggested by analogy to the properties of previously described colonization factors that these fimbriae may play a part in the colonization of the intestinal epithelium.

The ability of enterotoxigenic *E. coli* (ETEC) to adhere to the intestinal epithelium of both human and animals is an important factor in the pathogenesis of diarrheal disease. The adherence may be mediated by certain surface factors such as the fimbrial antigens K88 (16), K99 (25), 987P (15), CFA/I (13), and CFA/II (10). CFA/I and CFA/II are immunologically distinct, plasmid-controlled colonization factor antigens found in some ETEC strains isolated from humans. CFA/I causes mannose-resistant hemagglutination (MRHA) of human and bovine erythrocytes, whereas CFA/II causes MRHA of bovine erythrocytes only.

The incidence of CFA/I and CFA/II in ETEC strains varies among different studies (1, 24). In a retrospective study of diarrhea in travelers in Mexico, CFA/I was found in 86% of the strains (12). However, in a study by Cravioto et al. (4) of ETEC strains isolated from patients with diarrhea in 14 countries, CFA/I was found in 17% of the strains. Moreover, in volunteer challenge studies (17), four of six ETEC strains which caused diarrhea lacked CFA/I and CFA/II. Thus, it is likely that ETEC strains may possess adherence factors other than CFA/I and CFA/II which facilitate their attachment to the intestinal epithelium. In an attempt to identify such factors, we studied a large number of ETEC strains which lacked CFA/I and CFA/II but which were isolated from patients with diarrhea.

† Present address: Instituto National de Ciencias y Tecnologia de la Salud del Niño-DIF, Insurgentes Sur 3700, Mexico 22, D.F., Mexico.

MATERIALS AND METHODS

Bacterial strains. A total of 742 ETEC strains isolated in several countries from patients with diarrhea were examined. These strains were from the collection of the Division of Enteric Pathogens, Colindale, England, where they had been maintained on Dorset egg medium at room temperature. The infant mouse test (5) was used for the detection of heat-stable enterotoxin (ST). The Y1 adrenal cell (8) and the Chinese hamster ovary cell (14) tissue culture systems were used to detect heat-labile enterotoxin (LT).

The strains were identified by biochemical tests (3) and were serotyped with antisera for *E. coli* O groups O1 to O164 and for flagellar antigens H1 to H56 (22). The strains were also tested against an O antiserum prepared against strain E10702. This strain was isolated in Bangladesh from the feces of a patient with diarrhea, and it has been recognized by the Collaborative Centre for Reference and Research on Escherichia (World Health Organization) as the type strain for a new *E. coli* O group, O167 (I. Ørskov and F. Ørskov, personal communication).

Strains H10407 (O78:H11, CFA/I positive), H10407-P (O78:H11, CFA/I negative), PB-176 (O6:H16, CFA/II positive), and PB-176-P (O6:H16, CFA/II negative) were obtained from D. J. Evans (10, 13).

Media. For the production of fimbriae, the bacteria were grown on CFA agar, which contains Casamino Acids (1%), yeast extract (0.15%), magnesium sulfate

(0.005%), and manganese chloride (0.0005%) in 2% agar (11).

Hemagglutination. The strains were grown overnight at 37°C on CFA agar and suspended in 0.85% saline to a concentration of 2×10^9 bacteria per ml. The tests were performed on white porcelain tiles placed on ice and at room temperature with washed human group A, bovine, and guinea pig erythrocyte suspensions (9). MRHA was determined with erythrocyte suspensions containing 1% D-mannose.

Production of antisera. Antisera against *E. coli* strains H10407 and PB-176 were obtained by inoculating rabbits five times at 5-day intervals with increasing doses of a suspension in saline containing 4% formaldehyde. Blood (40 ml) was taken 5 and 10 days after the last injection, and the rabbits were exsanguinated after 15 days. The products of all three bleedings were pooled for use. Absorptions were carried out by mixing undiluted antiserum with a live culture at 50°C for 2 h until the serum was specific for the vaccine strain. Specific CFA/I and CFA/II antisera were prepared by absorbing the H10407 antiserum with strain H10407-P and absorbing the PB-176 antiserum with strain PB-176-P.

A specific antiserum for the fimbrial antigen of strain E8775 was prepared in a similar way by absorption with strain E8775-B, which is an MRHA-negative variant of E8775 that does not produce ST or LT.

Double immunodiffusion tests. Saline extracts of all of the strains were prepared at 60 and 100°C (23) for tests against the antisera for CFA/I and CFA/II by the Ouchterlony gel immunodiffusion technique (26). Extracts of strains H10407 and PB-176 were also tested against the absorbed antiserum specific for E8775.

Electron microscopy. Strains E8775 and E8775-B were grown on CFA agar at 37°C for 24 h and subcultured for another 24-h incubation period. Strain E8775 was also grown at 22°C. A thick suspension of the culture (ca. 10^{10} cells per ml) was made in peptone water containing 4% formaldehyde. The suspension was diluted 1/2, in distilled water, and 1 drop was applied to a Formvar-carbon-coated electron microscope grid (400 mesh) for 3 min. Excess fluid was removed by blotting with filter paper, and a drop of 1% phosphotungstic acid, pH 6.4, was placed on the grid for 1 min before excess fluid was blotted as before. For electron microscopy in which anti-E8775 serum was used, cultures were suspended in peptone water without formaldehyde. One drop of the suspension diluted 1/2 in distilled water was applied to the grid as before. Excess fluid was removed by blotting, and the grid was floated, sample side down, on a drop of antiserum diluted 1/30 in phosphate-buffered saline. The serum drops with grids were incubated in a humidified chamber for 15 min at room temperature. Grids were removed from the drops, blotted, washed four times with distilled water, stained for 1 min with 3% phosphotungstic acid, and finally blotted.

RESULTS

Survey of ETEC strains. All 742 strains were tested for MRHA of human and bovine erythrocytes and for CFA/I and CFA/II by using immunodiffusion. There was no difference between MRHA carried out at room temperature and MRHA carried out on ice. Table 1 shows the results of this survey. CFA/I was found in 91 (12%) of the strains, and CFA/II was found in 120 (16%) of the strains.

Of the 139 strains which caused MRHA of human and bovine erythrocytes, 91 were shown to produce CFA/I; the remaining 48 strains were not CFA/I positive and were investigated further. A serum raised against one of these strains, E8775 (O25:H42; ST and LT positive) was absorbed with E8775-B, a variant of E8775 which was MRHA negative and did not produce ST or LT. The 48 MRHA-positive, CFA/I-negative strains all reacted with this antiserum on slide agglutination and immunodiffusion. The serotypes of these strains, their toxin production, and their country of origin are shown in Table 2. The 742 ETEC strains examined in the survey were of 54 serogroups and were isolated in 14 countries. However, 50% of the strains came from Bangladesh and 8% came from Japan. The 48 strains positive with the E8775 antiserum were of the serotypes O25:H42, O25:H-, O115:H40, and O167:H5; they were isolated in Japan and Bangladesh from patients with diarrhea.

Characterization of strain E8775. Strain E8775 caused MRHA of human and bovine erythrocytes but did not react with CFA/I and CFA/II antisera. When an antiserum prepared by using E8775 as a vaccine was absorbed with the MRHA-negative variant E8775-B, immunodiffusion tests gave a precipitin line that was specific for E8775 extracts prepared at 60 and 100°C. This specific antiserum did not react with extracts of CFA/I or CFA/II.

Examination of strain E8775 by electron microscopy showed that 88% of the organisms were heavily fimbriate (Fig. 1a). The fimbriae were not type 1 fimbriae, since the strain did not cause mannose-sensitive hemagglutination of guinea pig erythrocytes. Incubation with specific antiserum against E8775 showed that the fimbriae were heavily coated with antibody. The MRHA-negative variant E8775-B lacked fimbriae, and no fimbriae were seen in the background (Fig. 1b). Examination of strain E8775 grown overnight at 22°C showed that 71% of the organisms lacked fimbriae.

DISCUSSION

In a survey of 742 ETEC strains belonging to 54 different serogroups and isolated from several countries, we found that 12% of the strains possessed CFA/I and 16% possessed CFA/II. These figures are rather low compared with those of previous studies, such as the study of travelers' diarrhea in Mexico, in which CFA/I was found on 86% of the strains (12), and another study (24) in which 16 of 77 ETEC strains were found to have CFA/I. Our results

TABLE 1. MRHA and identification of CFA/I and CFA/II in ETEC strains

MRHA pattern	Total no. of strains with indicated MRHA pattern	No. of CFA/I-positive strains	No. of CFA/II-positive strains	No. of CFA/I- and CFA/II-negative strains
Human only	1	0	0	1
Bovine only	129	0	120	9
Human and bovine	139	91	0	48
No MRHA	473	0	0	473

agreed more closely with those of Cravioto et al. (4), who found CFA/I in 17% of the ETEC strains isolated from patients with diarrhea in 14 countries. It should be noted that although the ETEC strains in the present survey were isolated in 14 countries, 50% of the strains were isolated in Bangladesh. No MRHA with human or bovine erythrocytes was given by 473 (64%) of the strains. This suggests that these strains may possess an adhesion mechanism other than that of CFA/I or CFA/II which cannot be detected by MRHA of human or bovine erythrocytes. These strains are being investigated by using blood of additional animals and other types of cells.

Forty-eight of the strains causing MRHA of human and bovine erythrocytes did not react with either CFA/I or CFA/II antiserum. An antiserum raised against one of these strains, strain E8775, was absorbed with an MRHA-negative variant and was used to test the 48 MRHA-positive, CFA-negative strains. All of these strains reacted with the E8775 antiserum on immunodiffusion.

Strain E8775 was isolated from a patient in Bangladesh suffering from diarrhea. The strain produced ST, LT, and a fimbrial surface antigen which was shown to be distinct from CFA/I and CFA/II by immunodiffusion and immunoelectron microscopy. The fimbriae had properties similar to those of previously identified adhesion factors found in ETEC, i.e., CFA/I, CFA/II, K88, and K99. These properties include MRHA activity correlated with the production of fimbriae and the loss of MRHA activity and fimbriae when the organism was grown at 22°C. The E8775 antigen was also similar to CFA/I and CFA/II in that it was restricted to strains of certain serogroups. CFA/I is restricted to ETEC strains of the O groups O15, O25, O63, O78, O126, O128, and O153; similarly, CFA/II is restricted to O6, O8, O9, O115, and O154. In the present study, ETEC strains from 54 serogroups were examined, and the E8775 antigen was found only on strains of the serogroups O25, O115, and O167.

The infant rabbit and the adult rabbit ligated intestinal loop were used to show colonization by ETEC strains possessing CFA/I or CFA/II (10, 13). Experiments in which these systems were used and human volunteer feeding experiments are required to evaluate the possible role of the fimbriae of E8775 in intestinal colonization by ETEC strains. Ørskov and Ørskov (24) have proposed that a special prefix, F, be used to describe the attachment fimbriae of K88, K99, 987P, CFA/I, and CFA/II. If the fimbriae of strain E8775 prove to be a new adhesion factor, then these fimbriae could be included in this system.

Recently Deneke et al. (6, 7) have identified three distinct pilus serotypes found on ETEC strains. Antisera raised against these pili reacted with 56% of ETEC strains from humans. Unlike the cases of CFA/I, CFA/II, and the fimbriae of strain E8775, no correlation between the anti-

TABLE 2. Survey of the serotypes of ETEC strains among which E8775-positive strains were found

Serotype	Country of origin	Enterotoxin produced	No. of strains tested	No. of strains with human and bovine MRHA patterns	No. of strains positive with the E8775 fimbrial antiserum
O25:H42	Bangladesh	ST	1	1	1
	Bangladesh	LT	1	1	1
	Bangladesh	ST, LT	20	20	20
	Japan	ST	8	6	6
O25:H-	Japan	ST, LT	4	3	3
O115:H40	Bangladesh	ST	4	1	1
	Bangladesh	ST, LT	7	5	5
O167:H5	Bangladesh	ST	16	11	11

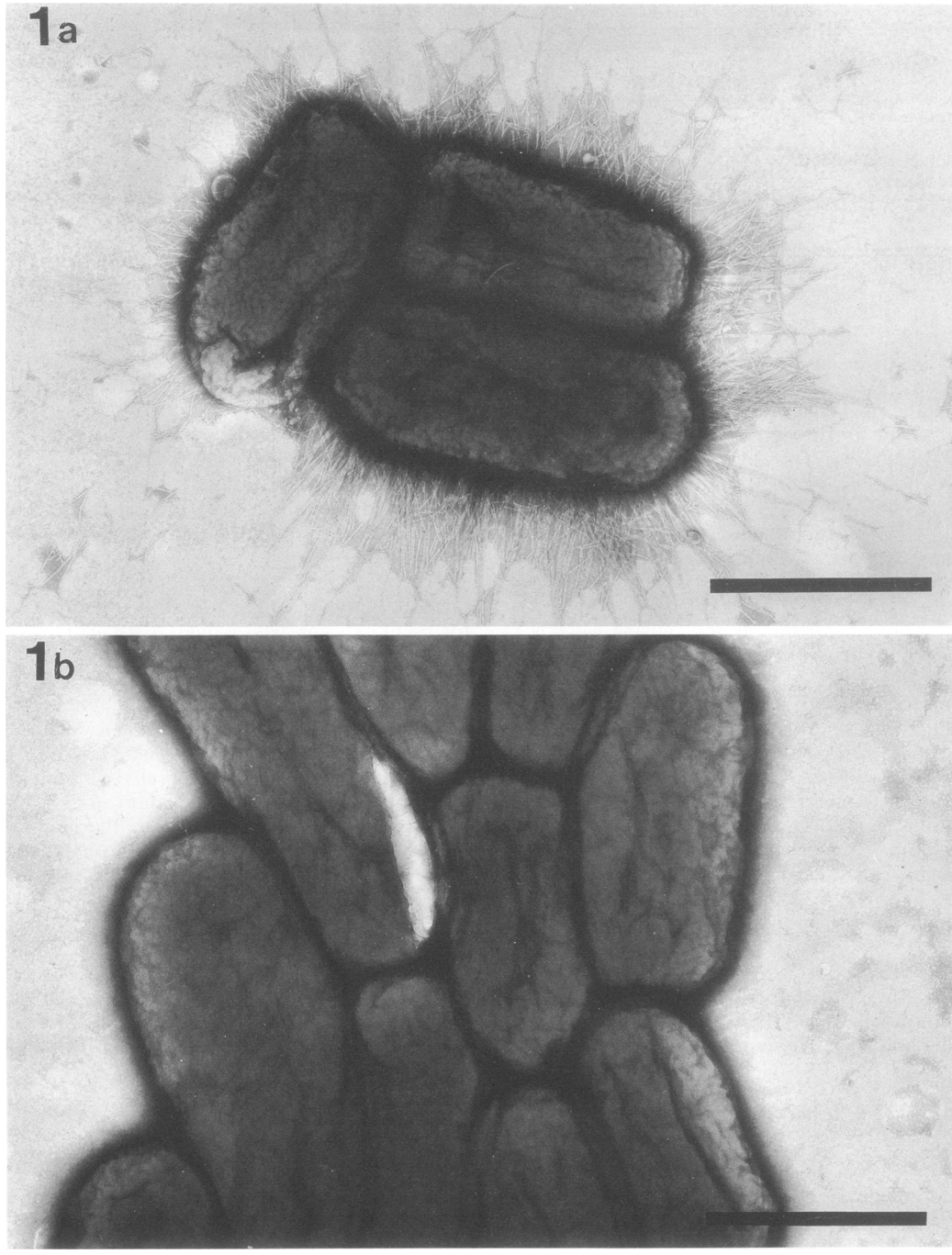

FIG. 1. Electron micrographs of negatively stained preparations of *E. coli* strain E8775 showing fimbriae (a) and MRHA-negative variant E8775-B, which does not possess fimbriae (b). Magnification, ×32,000. Bars, 1 μm.

genic types of these three pili and the O serogroups of the strains positive with the pilus antisera or the MRHA pattern of the pili with human, bovine, and guinea pig erythrocytes was found. The purified pili can bind to buccal cells, but neither the pili nor the strains possessing the pili were tested for adhesion to human or animal intestinal cells.

The ability to produce CFA/I or CFA/II may be lost or transferred from strains possessing

these factors, since the factors are plasmid controlled (13, 31). It has been shown that the genes coding for CFA/I and ST are on the same plasmid (18, 19, 31), and in one case, the same plasmid coded for CFA/I, ST, and LT. A plasmid coding for CFA/II, ST, and LT has been transferred into *E. coli* K-12 (27), and a similar transfer of a plasmid coding for CFA/II, ST, and LT from three strains of *E. coli* O6 has also been accomplished (H. R. Smith, unpublished data). Spontaneous variants of strain E8775 have been found which were MRHA negative and which had lost the ability to produce fimbriae, suggesting that the production of these fimbriae may also be plasmid controlled. Of the 48 ETEC strains which possessed the E8775 antigen, one strain produced LT only, 19 strains produced ST only, and 28 strains produced ST and LT. Four strains of the serogroup O25 (unpublished data), which had originally been isolated as enterotoxigenic strains, were later found to have lost the ability to produce ST and LT and yet were still MRHA positive and still reacted with the E8775 antiserum. This suggests that the genetic determinants for the production of the E8775 fimbriae may not be linked to those coding for ST and LT production, and this is being investigated at present.

Surveys in Bangladesh have shown that ETEC strains are a major cause of diarrhea and death in young children and adults (2, 29, 30), and it is possible that this diarrhea can be prevented by the use of a multivalent, purified fimbrial vaccine. Such a vaccine would prevent the adherence of the bacteria to the intestinal mucosa by stimulating local immunity to produce antibodies against the attachment fimbriae. This idea has been supported by work with ETEC strains pathogenic for animals: vaccination of pregnant cows and pigs with purified K88 (28, 32), K99 (20, 21), and 987P-type (20) fimbrial vaccines protected the offspring against otherwise lethal doses of ETEC strains bearing the homologous fimbrial antigen. For a fimbrial vaccine to be effective against ETEC strains causing diarrhea in humans, it is necessary to identify the range of fimbriae or other factors which enable colonization. The fimbriae of strain E8775 may represent such a new colonization factor.

ACKNOWLEDGMENTS

A.C. was supported by grant 1243 from the National Council of Science and Technology of Mexico (CONACYT).

We are grateful to Anne M. Field of the Virus Reference Laboratory, Central Public Health Laboratory, London, for the electron microscopy.

LITERATURE CITED

1. **Back, E., R. Mollby, B. Kaijser, G. Stintzing, T. Wadstrom, and D. Habte.** 1980. Enterotoxigenic *Escherichia coli* and other gram-negative bacteria of infantile diarrhea: surface antigens, hemagglutinins, colonization factor antigen, and loss of enterotoxigenicity. J. Infect. Dis. **142**:318–327.
2. **Black, R. E., M. H. Merson, A. S. M. M. Rahman, M. Yunus, A. R. M. A. Alim, I. Huq, R. H. Yolker, and G. T. Curlin.** 1980. A two year study of bacterial, viral and parasitic agents associated with diarrhea in rural Bangladesh. J. Infect. Dis. **142**:660–664.
3. **Cowan, S. T., and K. J. Steel.** 1965. Manual for the identification of medical bacteria. Cambridge University Press, Cambridge, England.
4. **Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe.** 1979. Mannose-resistant haemagglutination of human erythrocytes by strains of *Escherichia coli* from extraintestinal sources: lack of correlation with colonization factor antigen (CFA/I). FEMS Microbiol. Lett. **6**:41–44.
5. **Dean, A. G., Y.-C. Ching, R. G. Williams, and L. B. Harden.** 1972. Test for *Escherichia coli* enterotoxin using infant mice. Application in a study of diarrhea in children in Honolulu. J. Infect. Dis. **125**:407–411.
6. **Deneke, C. F., G. M. Thorne, and S. L. Gorbach.** 1979. Attachment pili from enterotoxigenic *Escherichia coli* pathogenic for humans. Infect. Immun. **26**:362–368.
7. **Deneke, C. F., G. M. Thorne, and S. L. Gorbach.** 1981. Serotypes of attachment pili of enterotoxigenic *Escherichia coli* isolated from humans. Infect. Immun. **32**:1254–1260.
8. **Donta, S. T., H. W. Moon, and S. C. Whipp.** 1974. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. Science **183**:334–336.
9. **Duguid, J. P., and R. R. Gillies.** 1957. Fimbriae and adhesive properties in dysentery bacilli. J. Pathol. Bacteriol. **74**:397–411.
10. **Evans, D. G., and D. J. Evans, Jr.** 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect. Immun. **21**:638–647.
11. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. **19**:727–736.
12. **Evans, D. G., D. J. Evans, Jr., W. S. Tjoa, and H. L. DuPont.** 1978. Detection and characterization of colonization factor of enterotoxigenic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. **19**:727–736.
13. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.
14. **Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman.** 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **10**:320–327.
15. **Isaacson, R. E., B. Nagy, and H. W. Moon.** 1977. Colonization of porcine small intestine by *Escherichia coli*: colonization and adhesion factors of pig enteropathogens that lack K88. J. Infect. Dis. **135**:531–539.
16. **Jones, G. W., and J. M. Rutter.** 1974. The association of K88 with hemagglutinating activity in porcine strains of *Escherichia coli*. J. Gen. Microbiol. **84**:135–144.
17. **Levine, M. M., M. B. Rennels, V. Daya, and T. P. Hughes.** 1980. Hemagglutination and colonization factors in enterotoxigenic and enteropathogenic *Escherichia coli* that cause diarrhea. J. Infect. Dis. **141**:733–737.
18. **McConnell, M. M., H. R. Smith, G. A. Willshaw, A. M. Field, and B. Rowe.** 1981. Plasmids coding for colonization factor antigen I and heat-stable enterotoxin production isolated from enterotoxigenic *Escherichia coli*: Infect. Immun. **32**:927–936.
19. **McConnell, M. M., H. R. Smith, G. A. Willshaw, S. M. Scotland, and B. Rowe.** 1980. Plasmids coding for heat-

labile enterotoxin production isolated from *Escherichia coli* 078: a comparison of their properties. J. Bacteriol. **143**:158–167.

20. **Morgan, R. L., R. E. Isaacson, H. W. Moon, C. C. Brinton, and C.-C. To.** 1978. Immunization of suckling pigs against enterotoxigenic *Escherichia coli*-induced diarrheal disease by vaccinating dams with purified 987 or K99 pili: protection correlates with pilus homology of vaccine and challenge. Infect. Immun. **22**:771–777.
21. **Morris, J. A., C. Wray, and W. J. Sojka.** 1980. Passive protection of lambs against enteropathogenic *Escherichia coli*: role of antibodies in serum and colostrum of dams vaccinated with K99 antigen. J. Med. Microbiol. **13**:265–271.
22. **Ørskov, F., and I. Ørskov.** 1975. *Escherichia coli* O: H serotypes isolated from human blood. Acta Pathol. Microbiol. Immunol. Sect. B. **83**:595–600.
23. **Ørskov, F., I. Ørskov, B. Jahn, and K. Jahn.** 1971. Immunoelectrophoretic patters of extracts from all O and K antigen test strains. Correlation with pathogenicity. Acta Pathol. Microbiol. Scand. Sect. B. **79**:142–152.
24. **Ørskov, I., and F. Ørskov.** 1977. Special O:K:H serotypes among enterotoxigenic *E. coli* strains from diarrhea in adults and children. Occurence of CF (colonization factor) antigen and of hemagglutinating abilities. Med. Microbiol. Immunol. **163**:99–110.
25. **Ørskov, I., F. Ørskov, H. W. Smith, and W. J. Sojka.** 1975. The establishment of K99, a thermolabile, transmissible *Escherichia coli* K antigen, previously called "K_{co}", possessed by calf and lamb enteropathogenic strains. Acta Pathol. Microbiol. Scand. Sect. B. **83**:31–36.
26. **Ouchterlony, O.** 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. Sect. B. **26**:507–515.
27. **Penaranda, M. E., M. B. Mann, D. G. Evans, and D. J. Evans, Jr.** 1980. Transfer of an ST:LT:CFA/II plasmid into *Escherichia coli* K-12 strain RR1 by co-transformation with PSC301 plasmid DNA. FEMS Microbiol. Lett. **8**:251–254.
28. **Rutter, J. M., and G. W. Jones.** 1973. Protection against enteric disease caused by *Escherichia coli*—a model for vaccination with a virulence determinant. Nature (London) **242**:531–532.
29. **Ryder, R. W., D. A. Sack, A. Z. Kapikian, J. C. McLaughlin, J. Chakrabarty, A. S. M. Rahman, M. H Merson, and J. G. Wells.** 1976. Enterotoxigenic *Escherichia coli* and reovirus-like agent in rural Bangladesh. Lancet **i**:659–662.
30. **Sack, D. A., J. C. McLaughlin, R. B. Sack, F. Ørskov, and I. Ørskov.** 1977. Enterotoxigenic *Escherichia coli* isolated from patients at a hospital in Dacca. J. Infect. Dis. **135**:275–286.
31. **Smith, H. R., A. Cravioto, G. A. Willshaw, M. M. McConnell, S. M. Scotland, R. J. Gross, and B. Rowe.** 1979. A plasmid coding for the production of colonisation factor antigen I and heat-stable enterotoxin in strains of *Escherichia coli* of serogroup O78. FEMS Microbiol. Lett. **6**:255–260.
32. **Smith, H. W., and M. A. Linggood.** 1971. The effect of antisera in protecting pigs against experimental *Escherichia coli* diarrhea and oedema disease. J. Med. Microbiol. **4**:487–493.