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THE TRANSMISSIBLE NATURE OF ENTEROTOXIN PRODUCTION IN A HUMAN ENTEROPATHOGENIC STRAIN OF *ESCHERICHIA COLI*

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PLATES XV AND XVI

IN a minority of the porcine enteropathogenic strains of *Escherichia coli* examined by Smith and Halls (1968) and Smith and Gyles (1970a), enterotoxin production was shown to be controlled by a transmissible plasmid designated "Ent". Ent⁺ organisms are recognised by the fact that they cause dilatation of ligated segments of pig small intestine into which they have been injected. Since little more than 60 tests can be performed in one pig, it is, therefore, possible to identify only those strains that transmit Ent with high efficiency. That many more of the strains examined by Smith and Halls and Smith and Gyles might be able to transmit their ability to produce enterotoxin, but at a rate too low to be detected, was suggested by the observation of Smith and Linggood (1970) that all except one of 78 porcine enterotoxigenic strains of *E. coli* possessed the genetic elements responsible for plasmid transmission, i.e., the sex, conjugation or transfer factors. They also found that 71 per cent. of human enteropathogenic strains of *E. coli*, compared with 30 per cent. of non-pathogenic strains, possessed these factors. Because of this high incidence in the human enteropathogenic strains and the fact that the enterotoxin of human strains is different from that of pig strains (Smith and Halls, 1967a and b; Smith and Gyles, 1970b), human strains were examined to see whether their enterotoxin production was transmissible; the results are reported in this paper.

Cultures of human enterotoxigenic strains of *E. coli* do not dilate ligated pig intestine. They do, however, dilate ligated rabbit intestine, but inconsistently (Taylor, Wilkins and Payne, 1961). More consistent results were obtained by Smith and Gyles (1970b), using sterile lysates of cultures, their so-called LT preparations. In view of this, LT preparations were used in the present work.

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MATERIALS AND METHODS

Transfer of ability to produce enterotoxin. This was performed by a modification of the method of Smith and Halls (1968). 0.2 ml of a broth culture of a human enterotoxigenic strain of *E. coli* (the donor strain) and the same volume of a nalidixic acid-resistant mutant (*nal^r*) of a *lac*⁻ strain of *E. coli* K12F⁻ (the recipient strain) were inoculated into 10 ml of nutrient broth, and the mixture was incubated at 37°C for 24 hr. The mixed culture was then diluted with 10 ml of broth and incubation continued for a further 24 hr. It was passaged twice in broth containing sodium nalidixate, 30 µg per ml, to eliminate donor organisms, and then once in plain broth; each passage was at 37°C for 24 hr. After confirmation that this culture, referred to as the "final mated culture", contained organisms of the recipient strain only, an LT preparation was made from it and tested in a ligated segment of rabbit intestine. If dilatation resulted, LT preparations were made from cultures of individual colonies obtained from the final mated culture and tested. Cultures yielding preparations that caused intestinal dilatation were designated by the number of the recipient strain, K12F⁻, followed by the number of the donor strain in brackets and the symbol Ent⁺; negative cultures were designated in the same manner, except that they were given the symbol Ent⁻. Sometimes, *nal^r* mutants of strains other than *E. coli* K12F⁻ were used as recipients.

LT preparations of E. coli strains. These were made by a modification of the method of Gyles and Barnum (1969). After incubation at 37°C for 24 hr, the bacterial growth from each Roux flask of tryptose blood agar base (Oxoid) containing 2 per cent. Bacto-casitone (Difco) and 0.2 per cent. glucose was washed off with 20 ml of distilled water and disintegrated ultrasonically. The resulting lysate was centrifuged for 30 min. at 3000 r.p.m. in the angle-head of an MSE Major centrifuge to remove bacterial debris and passed through a membrane filter of a.p.d. 450 nm. Neomycin and polymyxin, 100 µg of each per ml, were then added. When lysates were being screened only for ability to dilate ligated intestine, filtration was often omitted. They were tested in ligated segments of rabbit intestine in doses of 4 ml; the dose in pigs was 6 ml.

Ligated intestine technique. After Elizabethan collars had been applied to prevent coprophagy, young adult rabbits were starved for 24 hr. They were anaesthetised by intravenous administration of sodium pentobarbitone and the abdomen was opened. Any debris in the small intestine was washed out by injecting 20 ml of normal saline containing 100 µg each of neomycin and polymyxin per ml into its anterior portion. The small intestine was divided by means of string ligatures into eight segments approximately 10 cm long and separated from each other by smaller segments 4 cm long. The LT preparations were injected into the longer segments. The most anterior segment commenced 30 cm from the stomach and the most posterior one ended 10 cm from the caecum. The abdomen was then closed and the rabbits were allowed to recover from anaesthesia. They were killed 24 hr later and the presence or absence of dilatation of the segments and the volume and character of the contained fluid were recorded. The technique employed in pigs has been described fully elsewhere (Smith and Halls, 1967*a* and *b*).

Antisera. These were prepared by inoculating live cultures of *E. coli* intravenously into pigs, according to the method of Smith and Gyles (1970*a*). In neutralisation tests, 1-2 ml of antiserum was mixed with one dose of an LT preparation; after standing for 0.5-1.0 hr, the mixture was injected into a ligated intestinal segment.

Fertility-inhibiting (fi) character of transfer factors. This was assessed by transmitting an antibiotic-resistance determinant or Col into an F⁺ strain of *E. coli* K12 and then examining the strain for susceptibility to lysis by the F-specific phage, MS2 (Davis, Strauss and Sinsheimer, 1961).

RESULTS

Altogether, 27 human strains of *Escherichia coli* were examined to determine whether their ability to dilate ligated rabbit intestine could be transmitted to *E. coli* K12F⁻. Of these, 26 had been incriminated in different outbreaks

of diarrhoea in babies and belonged to serotypes generally believed to be enteropathogenic; O26:K60:H11 or - (7 strains), O55:K59:H6 or 7 (3 strains), O111:K58:H2 (2 strains), O119:K69:H6 (1 strain), O125:K70:H21 (4 strains), O126:K71:H2 (2 strains), O127:K63:H4 (1 strain) and O128:K67:H2 (6 strains). An additional strain, belonging to serotype O6:K13:H1, was included because it strongly dilated ligated rabbit intestine. LT preparations of *E. coli* K12F⁻ that had been grown with each of these 27 prospective donors, i.e., the final mated cultures, were tested in at least two ligated segments of intestine of different rabbits; all except one failed to produce dilatation. The one positive preparation strongly dilated both segments into which it had been injected, the volume of accumulated fluid being 13 and 16 ml. The results of testing LT preparations of colonies obtained from the final mated culture of this dilatation-positive preparation are illustrated in fig. 1. Seven colonies were examined in all, and LT preparations of cultures of six of them, designated K12F⁻(H19)Ent⁺, consistently dilated rabbit intestine; those of the seventh, designated K12F⁻(H19)Ent⁻, consistently failed to do so. For example, in tests carried out in six rabbits with an LT preparation from one of the Ent⁺ colonies the volume of fluid that accumulated in the injected segments was 6, 9, 10, 15, 27 and 32 ml; no fluid was noted in any of the six segments of the same rabbits into which LT preparations from the Ent⁻ colony had been injected. An LT preparation of a mixture of organisms from a culture of one of the Ent⁺ colonies and a culture of the Ent⁻ colony in the ratio of 1 to 4 produced only a very slight dilatation of a ligated intestinal segment when injected in the customary 4-ml dose. The Ent donor strain H19 was grown in mixed culture with K12F⁻ on a further three occasions; the LT preparations made from all three final mated cultures of K12F⁻ strongly dilated rabbit intestine. LT preparations of final mated cultures of six strains of *E. coli* other than K12F⁻ that had been grown in mixed culture with H19 failed to dilate rabbit intestine.

E. coli H19 belonged to serotype O26:K60:H11 and had been isolated in 1967 from the faeces of a baby suffering from diarrhoea. It was non-haemolytic and was not latently infected with bacterial viruses active on *E. coli* K12F⁻. LT preparations of H19 dilated segments of rabbit intestine to approximately the same degree as did those of K12F⁻(H19)Ent⁺. H19 possessed a streptomycin/sulphonamide R factor and a Col factor, both of which were, during the mixture-culture experiments, transmitted to all the K12F⁻ organisms, including those that did not receive Ent. Additional mating studies revealed that the R and Col factors in H19 were not linked to each other, and that H19 possessed at least two transfer factors, an *fi*⁻ one and an *fi*⁺ one. Several of the other human enteropathogenic strains examined had also transferred their R factors to all the organisms in the final mated cultures of K12F⁻.

The neutralising effect of antisera on the ability of LT preparations to dilate ligated rabbit intestine is illustrated in fig. 2. The activity of LT preparations of both K12F⁻(H19)Ent⁺ and H19 was neutralised by antiserum against K12F⁻(H19)Ent⁺. The activity of LT preparations of K12F⁻(H19)Ent⁺ was also neutralised by antiserum against H30, a human enteropathogenic

strain of *E. coli* belonging to the same serotype as H19, but not by antiserum against K12F-(P307)Ent⁺, P307 being a pig enterotoxigenic strain of *E. coli* of serotype O8:K87,88ab. In another rabbit, LT preparations of K12F-(P307)Ent⁺ were shown to be neutralised by the homologous antiserum but not by K12F-(H19)Ent⁺ antiserum.

LT preparations of K12F-(H19)Ent⁺ and of H19 did not dilate ligated pig intestine; these organisms therefore behaved like other human enteropathogenic strains. LT preparations of K12F-(P307)Ent⁺ did produce dilatation; their activity was neutralised by homologous antiserum but not by K12F-(H19)Ent⁺ antiserum.

The heat resistance of LT preparations of H19 and K12F-(H19)Ent⁺ was identical, their activity being partially destroyed at 65°C for 15 min., and completely destroyed at 80°C for 15 min.

DISCUSSION

There seems little doubt that *Escherichia coli* K12F⁻ acquired the ability to dilate ligated segments of rabbit intestine from *E. coli* H19 during their growth in mixed culture. The reciprocal neutralisation by specific antisera of the dilating substances or enterotoxins produced by K12F-(H19)Ent⁺ and by H19 strongly supports this view. The possibility that our mixed mating cultures of H19 and K12F⁻ had been contaminated with extraneous enterotoxin-producing organisms was most unlikely, since transmission was achieved at every one of four attempts. The results also indicated that the particular enterotoxin involved was typical of those produced by human enteropathogenic strains of *E. coli*, and that the H19 strain was not unusual in that it had, for example, acquired a porcine Ent plasmid under natural conditions; such plasmids can be introduced into human enteropathogenic strains experimentally (Smith and Halls, 1968). The most likely explanation of our results is that enterotoxin production in H19, like that in some porcine enteropathogenic strains, is controlled by a transmissible plasmid, and that we have transferred it to K12F⁻.

The results of testing mixtures of K12F-(H19)Ent⁺ and Ent⁻ organisms indicated that some 20 per cent. or more of K12F⁻ organisms in a culture would have to acquire Ent if the culture were to yield an LT preparation capable of dilating a ligated segment of rabbit intestine. It is clear from this that only human enteropathogenic strains capable of transmitting Ent with extremely high efficiency would be identified by the technique available at present. The fact that Ent was not linked to the R determinant or to Col in H19 indicated that selecting for these characteristics in mated cultures of K12F⁻ with any of the other prospective donors examined in the present studies would be unlikely to aid the isolation of Ent⁺ recipient organisms; as far as R determinants were concerned, this certainly proved to be the case. The finding by Smith and Linggood (1970) of transfer factors in 71 per cent. of human enteropathogenic strains of *E. coli*, none of which possessed characters then known to be transmissible, suggests, however, that the incidence of transmissible Ent in the strains we examined might be higher than that actually found.

ENTEROTOXIN TRANSMISSIBILITY IN *E. COLI*

FIG. 1.—Ligated segments of rabbit intestine after injection of LT preparations of *Escherichia coli*. The segments are identified according to the materials injected into them. K12F⁻(H19)Ent⁺ colony 1 and K12F⁻(H19)Ent⁻ colony 3 are two colonies of recipient strain K12F⁻ that were grown from organisms that did or did not receive Ent from donor strain H19 during mixed culture. 0 = Uninoculated segments. $\times 0.5$.

ENTEROTOXIN TRANSMISSIBILITY IN *E. COLI*

FIG. 2.—Ligated segments of rabbit intestine after injection of mixtures of LT preparations and antisera (AS) prepared against live *Escherichia coli* organisms. The segments are identified according to the materials injected into them. K12F⁻(H19)Ent⁺ and K12F⁻(P307)Ent⁺ are recipient strains of K12F⁻ that received Ent from the donor human strain H19 or the donor porcine strain P307. pre = Pre-immune serum; post = post-immune serum. 0 = Uninoculated segments. $\times 0.5$.

SUMMARY

Lysed preparations of a human enteropathogenic strain of *Escherichia coli*, belonging to serotype O26:K60:H11, dilate ligated segments of rabbit intestine. This property was transmitted to *E. coli* K12F⁻ in mixed culture experiments. The efficiency of transfer was high, and occurred independently of an R determinant and of Col. Transmission of the same property from a further 26 human enteropathogenic strains of *E. coli* could not be achieved in similar experiments.

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