The Transmissible Nature of the Genetic Factor in *Escherichia coli* that Controls Enterotoxin Production

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SUMMARY

A genetic factor (designated Ent) responsible for enterotoxin production in six of 51 enterotoxigenic strains of *Escherichia coli* of porcine origin could be transmitted to other strains of *E. coli* and to *Salmonella typhimurium* and *S. choleraesuis* by conjugation in mixed culture. A high proportion of organisms of the recipient strains in these cultures was found to have accepted Ent, which was probably a plasmid. The possession of Ent by an organism was a stable characteristic; no organisms were found to have lost it during either laboratory cultivation, acriflavine treatment, or residence in the alimentary tract of pigs and mice. Ent could be transmitted independently of F, R, Hly and K88 factors; the transfer factor responsible for its transmission closely resembled F and the transfer factors of *fi* + R factors and Hly. Ent⁺ organisms could not be differentiated from Ent⁻ ones, morphologically, culturally or antigenically. The oral administration of enterotoxin-containing bacteria-free culture fluid of an Ent⁺ strain of *E. coli* had no apparent ill effect on pigs, piglets and calves; neither did the fluid influence skin permeability. The transmission of Ent to *S. typhimurium* and *S. choleraesuis* did not affect their pathogenicity. Three of four piglets given Ent⁺ *E. coli* organisms developed diarrhoea; three given Ent⁻ organisms of the same strain did not.

INTRODUCTION

Smith & Halls (1967*b, c*) showed, by using modifications of the method of De & Chatterje (1953), that, with one exception, the serotypes of *Escherichia coli* associated with enteric disease in the pig dilated ligated segments of pig intestine, while other strains did not do so. They also showed that the factor which determined whether or not a strain produced dilatation was ability to produce an enterotoxin. For a strain to produce diarrhoea in the animal, however, it also had to be able to proliferate in the anterior small intestine. The exceptional serotype, O139:K82, is more commonly associated with oedema disease than with diarrhoea; only one of the 23 strains of this serotype examined by Smith & Halls (1967*b*) was positive in the ligated intestine test.

In view of the transmissibility of α haemolysin production in *Escherichia coli* (Smith & Halls, 1967*a*), it was thought that perhaps a transmissible genetic factor might also be responsible for enterotoxin production in the one O139:K82 strain able to produce it. Experiments indicated that this was indeed the case. The results of these experiments and others in which this genetic factor, termed Ent, was transmitted from enterotoxigenic strains of *E. coli* to other strains of this species as well as to

Salmonella typhimurium and *S. choleraesuis* are described in this paper. So are pathogenicity tests with Ent⁺ and Ent⁻ organisms and their cell-free culture fluids.

METHODS

Transfer of ability to produce enterotoxin. The method finally adopted to transfer enterotoxin-producing ability was the following. Nutrient broth (Oxoid, no. 2) in 10 ml. amounts was seeded with 0.02 ml. of a 24 hr broth culture of an enterotoxigenic strain of *Escherichia coli* and with 0.02 ml. of a similar culture of a streptomycin-resistant mutant of another strain. The former is referred to as the prospective donor and the latter as the prospective recipient strain. The mixed culture was incubated at 37° for 48 hr and then 0.2 ml. was inoculated into 10 ml. of broth containing streptomycin sulphate 50 µg./ml. After incubation at 37° for 24 hr, 0.1 ml. of this material was inoculated into another 10 ml. of the streptomycin-containing broth. This was incubated similarly and then subcultured in 0.02 ml. amount into 10 ml. of plain broth. After incubation at 37° for 24 hr, this culture, referred to as the passaged mating culture, was inoculated on to a suitable solid medium to confirm, as far as possible, that it contained only organisms of the prospective recipient strain. Whenever it was necessary to distinguish prospective recipient from donor strains, their growth characteristics, drug sensitivity, ability to produce haemolysin and, always, antigenic structure were utilized. If the results of these tests were satisfactory, the passaged mating culture, in 1.0 ml. amount and after the addition of 50 µg. streptomycin sulphate, was submitted to the ligated intestine test in a pig. If it produced dilatation it was subjected to two serial single-colony isolations on MacConkey agar and several single colony isolates were again examined by the ligated intestine test. Individual colonies were tested until at least one dilatation-positive culture was obtained. After confirming once again that the bacteria were those of the recipient strain, a representative number of dilatation-positive cultures, designated Ent⁺, and dilatation-negative cultures, designated Ent⁻, from each positive mating culture were freeze-dried and also stored at 5° on Dorset egg medium.

The above procedure was varied occasionally. For example, when the prospective donor strain was itself streptomycin-resistant, then an ampicillin-resistant or nalidixic acid-resistant mutant of the prospective recipient strain was used. The mating culture in this case was passaged through broth containing ampicillin or nalidixic acid, 30 µg./ml., instead of streptomycin. When the prospective recipient and donor strains had the same antigenic structure, a mutant of the recipient resistant to streptomycin and nalidixic acid was used for mating, the mating culture being passaged through broth containing streptomycin only. Very occasionally, a fully drug-sensitive prospective recipient strain was used. When this was a *Salmonella* the mating culture was passaged through selenite medium instead of drug-containing broth; when it was an *Escherichia coli*, colonies of the prospective recipient strain isolated from the passaged mating culture were tested directly in the ligated intestine, for it was useless to test the passaged mating culture since it would also contain the donor. With some positive passaged mating cultures, colonies were tested first in pools of five; the constituent colonies of any pool giving a positive reaction were then tested separately.

Cell-free enterotoxin-containing culture fluids. These were prepared by the methods of Smith & Halls (1967*c*) using either soft agar or liquid medium. After the culture fluids were separated from the bacteria by centrifugation they were held at 65° for

10 min. to kill the few bacteria that remained, and neomycin sulphate, 100 μ g./ml. was added, after which they were tested for enterotoxicity by injection in 15 ml. amounts into ligated segments of pig intestine.

Ligated intestinal preparations. The technique of performing ligated intestinal tests on different species of animals has been described fully elsewhere (Smith & Halls, 1967*b*). Unless otherwise stated, all the intestinal preparations in the present studies were made in pigs. Briefly, weaned pigs that had been starved for 24 hr were anaesthetized and their small intestine, beginning 1 m. from the pylorus and ending 3 m. from the caecum, was divided into segments 10–12 cm. long by string ligatures. Alternate segments were injected with the materials under test and the intermediate ones left as uninoculated controls. It was usually possible to perform 50 tests in each pig. The abdomen was then closed and the pigs 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 fluid they contained was recorded. Since the length of the segments was approximately constant, e.g. $\pm 25\%$, the volume of fluid recovered served as a measure of the severity of the reaction. In assessing this, however, the region of the intestine in which a test was made had to be taken into consideration because the anterior portion is more susceptible to dilatation than the posterior portion, susceptibility decreasing gradually from anterior to posterior. For this reason, materials to be compared were always tested in segments close to each other.

Consistent results are obtained when strains of *Escherichia coli* are tested by the procedure outlined above and positive segments are easily differentiated from negative ones (Smith & Halls, 1967*b, c*). Positive segments in the anterior test region contain 20–70 ml. of exudate and in the posterior test region seldom less than 10 ml.; by contrast negative segments contain 0.2–0.5 ml. of red amorphous semi-solid material. The anterior region was always used for testing cell-free culture fluids, the less reactive posterior region being unreliable for this purpose. Bacteria-free fluids from non-enterotoxigenic strains are usually completely absorbed from the segments into which they have been injected. Sometimes, however, a few ml. of thick mucoid fluid remained. By contrast, segments injected with culture fluids of enterotoxigenic strains were strongly dilated and might contain as much as 120 ml. exudate.

As mentioned previously, streptomycin was added to broth cultures of streptomycin-resistant organisms, and neomycin to all bacteria-free culture fluids, immediately before they were tested in the ligated intestine. The purpose of this was to suppress the multiplication of drug-sensitive *Escherichia coli* that might already be present in the segments; very occasionally dilatation of a control uninoculated segment occurred due to the multiplication in it of enterotoxigenic *E. coli*. Whenever a passaged mating culture or isolates from it were being tested, the corresponding prospective donor and recipient strains were always injected into nearby segments.

Detection of R and Hly factors. The presence of transmissible R factors (Watanabe, 1963) was detected by the methods of Smith (1966), and the presence of Hly factors by the methods of Smith & Halls (1967*a*).

*The *fi* character of Ent.* This was determined by introducing the Ent factor into an F⁺ culture of *Escherichia coli* K12 and then examining it for visible lysis by phage MS2 (Davis, Strauss & Sinsheimer, 1961). The validity of this test has been demonstrated by Meynell & Datta (1966).

Skin permeability. The effect of bacteria-free culture fluids on skin permeability was examined by the method of Craig (1965). The fluids were injected intradermally in 0.1 ml. into the shaven back of rabbits and into the ventral abdominal wall of pigs and calves. The Pontamine sky blue 6XB, 0.12 ml. of a 5% solution/100 g., was given intravenously 24 hr later.

RESULTS

The incidence of transmissible enterotoxin factor (Ent) amongst enterotoxigenic strains of Escherichia coli

Donors. Following the initial observation that strain P3, the only O139:K82 strain of *Escherichia coli* in our original collection to produce enterotoxin, could transfer this property to non-enterotoxigenic strains, a further 50 strains that had been incriminated in different outbreaks of diarrhoea in pigs were examined to determine whether their enterotoxigenic properties were also transmissible. Five of the 50 were positive. Two of the five, P18 and P103, had the antigenic formula O8:K87,88ab and two others, P16 and P19, the formula O9:K9; the latter two were the only O9:K9 strains examined. The remaining strain, P255, was an O139:K82 strain that did not belong to our original collection. Consequently, P255 and P3 were the only two of twenty-four O139:K82 strains that produced enterotoxin and with both strains its production was transmissible.

In strains of the following serotypes enterotoxin production could not be shown to be transmissible, O141:K85ac (4 strains), O141:K85ab → 85ac (9 strains), O141:K85ab,88ab (8 strains), O8:K87, 88ab (1 strain), O8:K87, 88ac (4 strains), O147:K89,88ac (3 strains), O45:K?,88ac (2 strains) and O138:K81 (8 strains). Neither was enterotoxin production transmissible by 6 strains of the Abbotstown type, a type not yet been given an internationally accepted formula.

Recipients. Every strain of *Escherichia coli* examined for ability to act as recipient was tested by using strain P3 as prospective donor of the Ent factor. Many strains were also tested with P16 and P103 as donors and a few with P18 and P19. The factor was transmitted to the following strains: (1) K12F⁻ and F⁺. (2) Five non-pathogenic strains isolated from the faeces of healthy pigs in different herds. (3) An O139:K82 strain, P104, incriminated in an outbreak of oedema disease. (4) An O18:K? strain, F11, responsible for an outbreak of bacteraemia in broiler fowl. (5) An O26:K60:H-strain, H1, from a case of neonatal diarrhoea in a human being. This strain had consistently dilated rabbit intestine (see Smith & Halls, 1967*b*). Only after mixed culture with P3, however, did it dilate pig intestine. The O139:K82 strain, P104, was probably the best recipient; the Ent factor was readily transmitted to it from all six donors. Dilatation of pig intestine was also achieved with bacteria-free culture fluids of strains P104, F11, H1, K12F⁻ and K12F⁺ into which Ent had been introduced.

Figure 1 illustrates a positive and a negative result obtained during the testing of passaged mating cultures in ligated pig intestine.

Despite repeated tests with one or more of the six donors it was impossible to transmit Ent to 14 strains of *Escherichia coli*. These included three non-pathogenic strains isolated from healthy pigs in different herds. They also included four O139:K82 strains, strains of the same serotype as the good recipient, P104. These strains had been tested using each of 3 known donors, P3, P16 and P103 on several occasions. Ten

passed mating cultures involving one of the four and P103 all yielded negative results. By contrast, 11 of 20 such cultures of P103 and P104 performed at the same time had yielded positive results.

*The transmission of Ent from Escherichia coli
to other bacterial species*

Attempts were made to transmit the Ent factor from the *Escherichia coli* strains P3, P16, P19 and P103 to 10 strains of *Salmonella typhimurium*, 10 strains of *Shigella flexneri*, two strains each of *S. dublin*, *Proteus vulgaris* and *P. morganii*, and one

Fig. 1. Ligated segments of pig intestine inoculated with cultures of *Escherichia coli*. The segments are identified according to the strains used for inoculating them. P3 and P103 = enterotoxigenic strains. P10 = non-enterotoxigenic strain. P10 (P3) and P10 (P103) = passaged mating cultures of P10 in which P3 and P103 had been employed as prospective donors. The ability to produce enterotoxin has been transferred to P10 from P3 but not from P103. O, uninoculated segments. $\times \frac{1}{3}$.

strain each of *S. choleraesuis*, *Shigella sonnei*, *Klebsiella aerogenes* and *Pseudomonas aeruginosa*. Transmission was achieved to three strains of *S. typhimurium*, and to the one strain of *S. choleraesuis*, but not to any of the other prospective recipients. It was transmitted to one strain of *S. typhimurium* on 11 of 14 occasions from *E. coli* P3, P16 and P19; the result of testing this strain before and after the introduction of Ent is illustrated in Fig. 2. By contrast seven passaged mating cultures of this strain with P103 as the prospective donor yielded negative results when tested in ligated pig intestine. After transfer to *E. coli* P104 from *S. typhimurium* Ent⁺ and *E. coli* F11 Ent⁺, the Ent factor retained its transmissible nature.

Since we were successful in transferring Ent to *Salmonella typhimurium*, we looked

for naturally occurring strains of this organism that would give a positive reaction in the ligated intestine test. Of 37 epidemiologically unrelated strains, 36 were negative. But one strain, 1652, produced some degree of dilatation which was less than that produced by strains of *S. typhimurium* to which Ent had been transferred from *Escherichia coli*. For example, the volume of exudate provoked by strain 1652 and a strain of *S. typhimurium* Ent⁺ in adjacent test segments in four different regions

Fig. 2. Ligated segments of pig intestine inoculated with cultures of *Salmonella typhimurium* and *Escherichia coli*. The segments are identified according to the strains used for inoculating them. P3E⁺ = enterotoxigenic strain of *E. coli*. TME⁻ = non-enterotoxigenic strain of *S. typhimurium*. TM(P3)E⁺ = enterotoxigenic strain of *S. typhimurium* obtained by mating P3 with TME⁻. O = uninoculated segments. $\times \frac{1}{2}$.

of the small intestine of a pig commencing in the more susceptible anterior part and ending in the less susceptible posterior part were, respectively, 19 and 30, 12 and 33, 0 and 29, and 0 and 12. It was not possible to transmit the ability to produce dilatation from 1652 to *S. typhimurium* or to *E. coli*.

The extent of Ent transfer in passaged mating cultures

From every passaged mating culture which dilated ligated pig intestine at least one colony of the recipient strain that had acquired Ent was obtained. The results of examining individual colonies from passaged mating cultures are shown in Table 1.

The incidence of Ent⁺ organisms in these cultures varied from 2 to 87 %. Only five colonies of *Escherichia coli* K12F⁻ were tested after mating with *E. coli* P3; all produced dilatation.

Before classifying a colony as Ent⁺ or Ent⁻ in the tests referred to above, it was tested in the ligated intestine of at least three pigs; consistent results were obtained. Control cultures of the prospective donor and recipient strains were always tested together with the passaged mating cultures and colonies obtained from them and they, too, yielded consistent results. Two of the good recipient strains, *Escherichia coli* P104 and a *Salmonella typhimurium* strain, were tested 70 and 35 times, respectively, during the present studies; they failed to dilate ligated pig intestine on every occasion. Cultures of 100 colonies from each of these two strains and also from each of another two strains of *E. coli*, P30 and F11, that were also capable of accepting Ent, were tested in pools of five in 10 pigs. A further 25 colonies obtained from the *S. typhimurium* strain were tested singly. Dilatation was never produced.

Table 1. *The incidence of Ent⁺ recipient organisms in passaged mating cultures*

Passaged mating culture		Colonies of recipient strain obtained from passaged mating culture	
Recipient	Donor	No. tested	% Ent ⁺
<i>E. coli</i> P104	<i>E. coli</i> P16	10	70
<i>E. coli</i> P104	<i>E. coli</i> P18	15	40
<i>E. coli</i> P104	<i>E. coli</i> P103	20	50
<i>E. coli</i> P104	<i>E. coli</i> P103	18	50
<i>E. coli</i> P20	<i>E. coli</i> P3	20	80
<i>E. coli</i> P30	<i>E. coli</i> P3	50	6
<i>E. coli</i> P30	<i>E. coli</i> P16	20	5
<i>E. coli</i> P66	<i>E. coli</i> P103	15	53
<i>E. coli</i> F11	<i>E. coli</i> P3	25	12
<i>E. coli</i> H1	<i>E. coli</i> P3	50	2
<i>S. typhimurium</i> strain 1	<i>E. coli</i> P3	15	67
<i>S. typhimurium</i> strain 1	<i>E. coli</i> P3	18	33
<i>S. typhimurium</i> strain 1	<i>E. coli</i> P3	18	22
<i>S. typhimurium</i> strain 1	<i>E. coli</i> P16	15	87
<i>S. typhimurium</i> strain 1	<i>E. coli</i> P19	10	60
<i>S. typhimurium</i> strain 1	<i>E. coli</i> P19	10	50
<i>S. typhimurium</i> strain 2	<i>E. coli</i> P3	25	36
<i>S. choleraesuis</i>	<i>E. coli</i> P3	10	60

P20, P30 and P66 were non-pathogenic strains of *E. coli* isolated from the faeces of healthy pigs.

The comparative dilating ability of different bacterial strains into which Ent had been introduced

Although detailed studies were not made, the general impression gained was that the degree of dilatation produced by a given strain into which Ent had been introduced was more a function of that strain than of the Ent factor or the strain which had donated it. For example, there was no important difference between the amount of fluid that accumulated in segments of pig intestine which had been injected with strains of *Escherichia coli* P104 into which Ent had been introduced from strains P3, P16, P18, P19, P103 or P255. The amount was usually in the region of 20 ml. when

tests were made in the anterior small intestine. Similar volumes were usually provoked by P3, the donor strain of the same serotype as P104. Greater volumes were commonly provoked by the other donor strains P16, P18 and P103; P255 was not tested often enough to permit any conclusions being drawn. In contrast to the Ent⁺ derivatives of P104, those of *Salmonella typhimurium* that had received the factor from P3, P16 or P19 produced much greater dilatation, the volumes provoked in the anterior intestine being 30–50 ml. irrespective of the donor strain involved. The *E. coli* strains K12F⁻Ent⁺ and K12F⁺Ent⁺ were the poorest dilators. These strains usually had to be injected in greater numbers than other strains to produce dilatation, and then the volume of exudate provoked was only 3–10 ml. The reason for this was probably poor survival of the bacteria in the ligated segments, probably because K12 strains have been maintained in the laboratory away from their natural habitat for many years. Support for this view was provided by the observation that bacteria-free culture fluids of these strains produced dilatation of ligated segments as strongly as culture fluids of the other Ent⁺ strains (Fig. 3). As an example of the dilatation produced by culture fluids, when culture fluids of P104 into which Ent had been introduced from P3 were tested in seven segments in one pig, the volume of exudate that accumulated in these segments was 40–90 ml. (median 50 ml.); the volumes in segments injected with culture fluids of P104 itself was 2–9 ml. (median 5 ml.).

The stability of Ent in bacteria; the effect of acriflavine

All the *Escherichia coli* and *Salmonella* strains to which Ent had been transmitted were maintained on Dorset egg medium at 5°. Between the time of inoculation and the completion of the work described in this paper (5–12 months) they were examined on many occasions. Their ability to dilate ligated pig intestine remained constant. Apart from periodic subculture, five of the six wild strains of *E. coli* from which Ent was transmitted had been maintained on Dorset egg medium for 6 years before they were examined.

Three strains of *Escherichia coli*, K12F⁻, H1 and F11, and one strain of *Salmonella typhimurium* to which Ent had been transmitted, were passaged daily for 10 days in nutrient broth at 37° by using small inocula transferred with the tip of a platinum wire. Five colonies from the final subculture of each strain were tested in ligated pig intestine; all produced dilatation.

No loss of Ent was detected from *Escherichia coli* or *Salmonella* organisms during residence in experimentally infected mice and pigs. The period between infection and recovery of *S. typhimurium* Ent⁺ and *S. choleraesuis* Ent⁺ in mice and/or pigs was 5–17 days. In the case of *E. coli* P104 Ent⁺ given to two pigs by mouth it was 63 days. *Escherichia coli* Ent⁻ and *Salmonella* given to pigs and/or mice did not acquire the ability to produce enterotoxin.

Strains of *Salmonella typhimurium* and *Escherichia coli* P104 to which Ent had been transmitted from *E. coli* P3 were incubated at 37° for 24 hr in nutrient broth containing acriflavine at a concentration which had a marked depressant effect on their growth rate. Twenty-five colonies from each of the resulting cultures were tested in ligated pig intestine; none had lost the ability to produce dilatation.

*The significance of culture filtrates, phages, colicines,
F, R, Hly and K88 factors in Ent transfer*

Culture filtrates. Ten passaged mating cultures were prepared in the usual manner except that filtrates, passed through a membrane of average pore diameter 250 m μ in 5–10 ml. amounts, of 24 hr nutrient broth cultures of the donor strains of *Escherichia coli*, P3, P16, P18, P19, P103 and P255, were used instead of the donor organisms themselves. The prospective recipient strain used in each mating culture was one or other of four strains of *E. coli*, P104, P30, H1 and F11, or a *Salmonella typhimurium* strain of proven recipient ability. None of the 10 cultures dilated ligated pig intestine.

Phages and colicines. Cultures of the six strains that were able to transmit Ent and of six whose enterotoxigenic character was not transmissible were tested for phage or colicine production with cultures of six strains that were able to receive Ent and six that were not. A strain of *Salmonella typhimurium* was included amongst the strains in each of the last two categories; the remaining 22 strains were of *Escherichia coli*. Five strains before and after they had received Ent were also tested against each other. Apart from the fact that one of the six strains that could not act as a recipient exhibited strong colicine action against 11 of the 12 strains which had been tested as donors, no phage or colicine activity was observed. Neither was any activity between the donor strains and the four strains of *E. coli* that were of similar antigenic structure to P104, the good recipient, but were unable to receive Ent.

F factor. As mentioned previously, Ent was transmitted to an *Escherichia coli* K12F⁻ strain and to a K12F⁺ strain. No more difficulty was experienced in transmitting it to the K12F⁺ strain than to the K12F⁻ strain. The K12F⁺ strain was no longer macroscopically lysed by the F specific phage MS2 after the introduction of Ent indicating that Ent was an *fi*⁺ factor (fertility inhibiting; Watanabe *et al.* 1964). In the same way as *fi*⁺ R factors (Meynell & Datta, 1966) and the Hly factor, Ent itself carried the gene for production of an F-like pilus, because F-specific phage, which uses the pilus as receptor, was able to multiply in cultures of K12F⁻Ent⁺ bacteria (Dr Elinor Meynell, personal communication).

R factors. Of the six strains of *Escherichia coli* shown to be able to transmit Ent, one, P18, contained R factors. The other five strains, as well as the 10 strains of *E. coli* and three *Salmonella* strains that were able to receive Ent, did not. Of four Ent⁺ colonies of *E. coli* P104 obtained from a passaged mating culture in which P18 was the donor, all four contained R factors. Of four Ent⁻ colonies from the same mating culture, two contained R factors and two did not.

Different R factors in four strains of *Escherichia coli* were transmitted to six strains of *E. coli* and one strain of *Salmonella typhimurium* into which Ent had already been introduced and into their Ent⁻ parent strains. There was no obvious difference in the rate of transmission of R factors into the Ent⁺ and Ent⁻ form of each of the seven strains. Dr E. S. Anderson (Enteric Reference Laboratory, Colindale, London) examined a *S. typhimurium* strain and the K12F⁻ strain for the presence of a transfer factor by his resistance determinant mobilization test (Anderson, 1965) before and after Ent had been transmitted to them. No transfer factor could be demonstrated in the two parent strains, which were Ent⁻, whereas both Ent⁺ derivatives carried one. As this test depends on mobilization of a determinant for streptomycin and sulphonamide resistance, its success indicates that the transfer factor concerned

can carry genetic determinants for both enterotoxin production and drug resistance.

Hly factor. Three of the six strains of *Escherichia coli* that were able to transmit Ent produced α haemolysin and so did one of the 10 strains of *E. coli* that were able to receive it. In none of these four strains was haemolysin production transmissible, as with the Hly factor (Smith & Halls, 1967*a*). All the 45 strains from which Ent was not transmitted produced α haemolysin, but in only seven strains was its production shown to be controlled by the transmissible Hly factor. Five of these seven also carried R factors. R factors alone were demonstrated in another eight of the 45 strains. One of the Hly⁺ R⁺ strains also possessed the K 88 factor (see below).

Fig. 3. Ligated segments of pig intestine inoculated with bacteria-free culture fluids of *Escherichia coli*. The segments are identified according to the strains from which the culture fluids were obtained. F⁻E⁻ and F⁺E⁻ = non-enterotoxigenic strains $\kappa 12$ F⁻ and $\kappa 12$ F⁺. F⁻E⁺ and F⁺E⁺ = enterotoxigenic strains of $\kappa 12$ F⁻ and F⁺ obtained by mating with the enterotoxigenic strain P3. O = uninoculated segments. $\times \frac{1}{2}$.

The Hly factor was introduced without difficulty from two Hly⁺ strains of *Escherichia coli* into four Ent⁺ strains of *E. coli*, two of these recipient strains being derived from *E. coli* $\kappa 12$ F⁻ and F⁺. During these mating procedures, R factors present in one of these two Hly⁺ donor strains was also transmitted to some of the recipient organisms. Some $\kappa 12$ F⁺ organisms, for example, contained as a result R, Hly and Ent factors.

The two Hly⁺ donor strains were also enterotoxigenic but passaged mating cultures of strains of *E. coli* able to receive Ent with these two strains as prospective donors did not dilate ligated pig intestine.

K88 factor. Four different strains of *Escherichia coli* into which the genetic elements controlling the production of K88 antigen (Ørskov & Ørskov, 1966) had been transmitted were tested in ligated pig intestine. One of these K88-containing strains had been prepared in this laboratory; the other three were received from Dr Ida Ørskov. All four strains failed to cause dilatation. The donor strains used in the preparation of these four strains were all enterotoxigenic, as are many strains of *E. coli* which produce the K88 antigen. Only two of the six strains that were able to transmit Ent (P18, P103) possessed K88 antigen. None of the strains to which they had transmitted Ent were found to have received this antigen.

Fig. 4. Ligated segments of calf intestine inoculated with cultures of *Escherichia coli* and *Salmonella choleraesuis*. The segments are identified according to the strains used for inoculating them. P66 (P103) E⁺ and E⁻ = enterotoxigenic and non-enterotoxigenic strains obtained from a mating of the non-enterotoxigenic *E. coli* P66, with the enterotoxigenic *E. coli* P103. P104 (P18) E⁺ and E⁻ = enterotoxigenic and non-enterotoxigenic strains obtained from a mating of the non-enterotoxigenic *E. coli* P104 with the enterotoxigenic *E. coli* P18. SC-S (P3) E⁺ and E⁻ = enterotoxigenic and non-enterotoxigenic strains obtained from a mating of a non-enterotoxigenic strain of *S. cholerae-suis* with the enterotoxigenic *E. coli* P3. O = uninoculated segments. $\times \frac{1}{3}$.

The effect of Ent⁺ bacteria on the ligated intestine of calves, rabbits and mice

Although ligated pig intestine was used to determine the Ent character of bacterial cultures in all the studies referred to previously, some cultures were also tested in the ligated intestine of other animal species.

Calves. With two calves, six strains of *Escherichia coli* into which Ent had been introduced were tested and all produced dilatation of intestinal segments. Similar

results were obtained with a *Salmonella typhimurium* Ent⁺ strain and the *S. choleraesuis* Ent⁺ strain. A portion of the ligated intestine of one of the two calves is illustrated in Fig. 4.

Rabbits. Two strains of *Salmonella typhimurium* which had received Ent from one or other of two donor strains and the *S. choleraesuis* Ent⁺ strain failed to dilate ligated intestine, so did the two donor strains concerned, P₃ and P₁₆.

Mice. With 10 mice, *Escherichia coli* P₁₀₄, a strain of *Salmonella typhimurium* and *S. choleraesuis*, all of which had received Ent from *E. coli* P₃, and P₃ itself, were submitted to the ligated intestine test. Negative results were obtained.

The distinguishing features of Ent⁺ and Ent⁻ organisms

Cultures of colonies obtained from dilatation-positive passaged mating cultures and classified as Ent⁺ or Ent⁻ according to their reaction in ligated pig intestine were examined to see whether the two kinds of cultures differed in other respects, especially in their pathogenicity. Strain *Escherichia coli* P₁₀₄ to which Ent had been transmitted from *E. coli* P₃ was frequently used in these studies because strains P₁₀₄ and P₃ were both of the same serotype, O₁₃₉:K₈₂. Another reason for using this strain P₁₀₄ was the possibility that it might be able to proliferate in the anterior small intestine of the intact pig by virtue of the fact that its parent had been isolated from a case of oedema disease. This would be of major significance in infection studies. All the other strains of *E. coli* to which Ent had been transmitted were non-pathogenic for the pig.

Cultural characteristics. The morphology on ordinary laboratory media of colonies of Ent⁺ and Ent⁻ organisms obtained from the same passaged mating culture appeared identical. Their microscopical appearance and their general cultural properties, too, were similar.

Antigenicity. Antisera were prepared in six rabbits by the multiple intravenous injection of live broth cultures of one or other of three different strains of *Escherichia coli* into which Ent had been introduced from *E. coli* P₃ or of bacteria-free enterotoxin-containing culture fluids of one or other of these three strains. Each antiserum was then absorbed with suspensions of the corresponding Ent⁻ organisms. All the absorbed antisera failed to agglutinate suspensions of homologous and heterologous Ent⁺ organisms, live or heat-treated to destroy their K antigens. The unabsorbed antisera also failed to agglutinate suspensions of Ent⁺ strains of *E. coli* and *Salmonella* except those for which they were known to contain O or K antibodies.

No antigen-antibody reactions were detected in plate precipitin tests (Ouchterlony, 1949) between extracts of Ent⁺ organisms or their enterotoxin-containing culture fluids and the absorbed antisera referred to above.

Antisera were prepared in six pigs by multiple intravenous injection of bacteria-free enterotoxin-containing culture fluids of *Escherichia coli* P₃, P₁₆ or P₁₈, three of the wild Ent⁺ strains, or by the parenteral administration of suspensions of live organisms of these strains. The addition of 10 ml. antiserum to 15 ml. bacteria-free culture fluid of the strain against which each one was prepared and to fluids of other enterotoxigenic strains had no effect on the size of the dilatation the fluids produced in ligated pig intestine.

Pathogenicity of live cultures to piglets. Seven piglets which had been removed from their mother before consuming colostrum were given 40 ml. of sterile cow's

milk, by stomach tube, before they were 4 hr old. Immediately, four were inoculated orally with 5×10^9 viable organisms of *Escherichia coli* P104 to which Ent had been transmitted from *E. coli* P3. The remaining three piglets were inoculated in a similar manner with P104 itself. Subsequently, all piglets were given 40–50 ml. of the cow's milk 3–5 times daily by stomach tube. Of the 4 given the Ent⁺ strain, one developed severe diarrhoea 11 hr after inoculation, it was killed 9 hr later when still suffering from diarrhoea. The number of viable organisms of the inoculating strain in the contents of its anterior small intestine was, $\log_{10}$ per ml., 7.2. Another developed severe diarrhoea at 30 hr and was killed when near death at 38 hr. The condition of a third piglet in this Ent⁺ group began to deteriorate rapidly at 40 hr and at 44 hr, when it was killed, it had developed severe diarrhoea. The viable counts of the inoculating strain in the anterior small intestinal contents of both these piglets were relatively low, $\log_{10}$ per ml., 5.3 and 5.2, respectively. No organisms were found in the internal organs of either of these three piglets. The remaining piglet in this group remained healthy as did all three piglets given P104.

Two groups of 4 pigs aged 8 weeks were inoculated orally with approximately 10^{10} viable organisms of *Salmonella choleraesuis*. One group was given an Ent⁺ strain and the other an Ent⁻ one, both being obtained from the same mating of a strain of *S. choleraesuis* with *Escherichia coli* P3. The clinical signs subsequently exhibited by both groups were identical. All eight pigs developed a severe diarrhoea, of similar intensity and duration, 3 days after inoculation.

Pathogenicity of live cultures to calves. Two 2-day-old colostrum-fed calves were given, by mouth, 10^{10} viable organisms of *Salmonella typhimurium* Ent⁺ and another two a similar dose of *S. choleraesuis* Ent⁺. The four calves died 3–5 days later, the principal lesion in all of them being a severe inflammation of the small intestine with necrosis of the ileum. Diarrhoea was not a prominent clinical sign. The course of the disease was similar to that observed in calves infected with Ent⁻ forms of these two *Salmonella* types.

Pathogenicity of live cultures to mice. Ent⁺ and Ent⁻ colonies obtained from matings of a *Salmonella typhimurium* strain with *Escherichia coli* P3, P16 and P19 were administered orally in doses of 10^9 viable organisms to groups of mice. The mortality pattern was similar in all groups indicating no difference in virulence between the Ent⁺ cultures and the Ent⁻ ones. Diarrhoea was no more common in mice given Ent⁺ organisms than in mice given Ent⁻ organisms. The mouse-virulence of *S. choleraesuis* also, was not altered as a result of accepting Ent. Neither was its virulence for rabbits.

Pathogenicity of bacteria-free culture fluids to pigs. Ten piglets were removed from their mothers before they had consumed colostrum and each one given, by stomach tube, 50 ml. of bacteria-free culture fluid of *Escherichia coli* P104 to which Ent had been transmitted from *E. coli* P3. Another 10 piglets were treated similarly except that the fluid they were given had been prepared from an Ent⁻ strain of P104. The 20 piglets were returned to their mothers 2½ hr later. All remained healthy. So did four 11-week-old pigs that were starved for 48 hr and then given 1000–1500 ml. of the bacteria-free culture fluid of *E. coli* P104 Ent⁺ by stomach tube.

Pathogenicity of culture fluids to calves. Two 1-day-old calves that had received no food were given 1500 ml. of the bacteria-free culture fluid of *Escherichia coli* P104 Ent⁺ by mouth. They showed no signs of ill-health as a result.

Pathogenicity of culture fluids to mice. Bacteria-free culture fluids, in 0.5 ml. amounts,

of three strains of *Escherichia coli* into which Ent had been transmitted from *E. coli* p3 were each injected intravenously into 2 mice. All 6 mice remained well.

Skin permeability. No difference was noted in pigs, calves and rabbits between the skin reactions produced by bacteria-free culture fluids of *Escherichia coli* P104 Ent⁺ and Ent⁻.

DISCUSSION

The results indicate that the ability to produce enterotoxin can be transmitted from some strains of *Escherichia coli* to others and to *Salmonella* strains and that transmission when it occurs is probably by conjugation. The available evidence suggests that, as in the case of the R, col, K88 and Hly factors, the actual production of enterotoxin in the donor strains is governed by an extrachromosomal genetic element, or plasmid, which has conjugation factor activity. The name Ent is proposed for this element which evidently belongs to the same category of sex factors as F, *fi*⁺ R factors and Hly which all determine the production of an F-type pilus subject to the same genetic regulation of function (Lawn, Meynell, Meynell & Datta, 1967).

No exclusion of the kind described by Scaife & Gross (1962) to occur between F factors was found to exist between Ent and F, R and Hly factors. They could be carried together in the same bacterium; all four were present in one of our laboratory strains of *Escherichia coli* K12 and a wild strain was found to contain R, Hly and the K88 factor, all transmissible to a K12F⁺ strain. When more than one was present in a strain, they were sometimes transmitted together, sometimes separately. Dr E. S. Anderson has utilized the transfer factor of Ent from the K12F⁻ Ent⁺ strain and from one of the *Salmonella typhimurium* Ent⁺ strains to mobilize R determinants (Anderson, 1965). It may well be that there is no specific relationship between a transfer factor and a particular plasmid. The transfer factor merely potentiates conjugation without exercising any control on the plasmids that are transmitted during the process.

Although the enterotoxin of many pig strains of *Escherichia coli* appeared to be similar (Smith & Halls, 1967*b, c*) most of the strains could not transmit the character. There are several possible reasons for this: the genes may be integrated in the chromosome; if plasmids are responsible, transfer factor activity may be absent; finally, the plasmids may be highly repressed like some R factors (Meynell & Datta, 1966). The only means of identifying an enterotoxigenic strain is the ligated intestine test, which severely limits experiments to distinguish between these possibilities. Not only does it restrict the number of tests that can be performed but the test itself becomes insensitive when enterotoxigenic organisms injected into a ligated segment are greatly outnumbered by non-enterotoxigenic ones (Smith & Halls, 1967*b*). This means that only those passaged mating cultures containing a comparatively high proportion of Ent⁺ recipient organisms would be identified; in fact, the lowest proportion found in the present studies was 2%. It is probable then that if suitable techniques had been available more strains could have been identified as donors of their enterotoxin production.

After its introduction into organisms of *Escherichia coli*, *Salmonella typhimurium* and *S. choleraesuis* Ent behaved as a stable character. No organisms were found to have lost it *in vivo* or *in vitro*, even after acriflavine treatment. In view of this stability and the fact that a high proportion of organisms of some of the strains of non-pathogenic *E. coli* isolated from pig faeces that were used as recipients in mating cultures accepted Ent, a substantial degree of transfer might be expected to have occurred

in nature as has happened in the case of R factors; Smith & Halls (1967*b*), did not find this to be so. All the strains of *E. coli* they examined which were not incriminated in outbreaks of diarrhoea in pigs did not dilate pig intestine. It may well be that, unlike the profound influence the widespread use of antibiotics has had on the emergence of R factor-containing bacterial populations, no comparable influences exist to favour the selection of Ent⁺ organisms. The possession of Ent alone may be of little selective advantage; the selection advantage possessed by enteropathogenic *E. coli* organisms probably resides principally in their ability to proliferate in the anterior small intestine.

The fact that three of the four piglets given *Escherichia coli* P104 into which Ent had been introduced developed diarrhoea, whereas all three given P104 itself remained healthy, supports the view that enterotoxin is responsible for the production of diarrhoea in the intact animal. In two of the piglets that had diarrhoea, however, the numbers of the infecting organisms in the anterior small intestine when they were killed was lower than that usually found in piglets suffering from *E. coli* diarrhoea. In view of this and the fact that the number of animals involved was small the evidence obtained from this experiment is suggestive rather than conclusive. Following its isolation from a case of oedema disease, the P104 strain had been maintained in the laboratory for some 12 years before it was used in the present work; perhaps during this period its ability to proliferate in the small intestine had become impaired. It was not possible to repeat this experiment with more recently isolated non-enterotoxigenic pathogenic strains because of our inability to introduce Ent into any of them. The failure of enterotoxin-containing bacteria-free culture fluids of *E. coli* to produce diarrhoea in the newly born piglets after its administration by mouth is probably explained by the fact that, even in ligated intestine studies, it is only the anterior small intestine that is susceptible to the dilating action of enterotoxin. In the piglets the fluid would not be expected to be retained for long in this anterior region and any fluid disturbance it might cause there would be corrected by absorption in the more posterior region. The situation is quite different from that occurring in ligated intestine studies where the injected enterotoxin-containing fluid is trapped for a long time in an isolated intestinal segment. It is also quite different from that which occurs in *E. coli* infection in the intact animal where enterotoxin is being continuously produced by large numbers of bacteria closely adherent to the mucous membrane.

The introduction of Ent into *Salmonella typhimurium* and *S. choleraesuis* did not enhance their pathogenicity for mice, rabbits, calves and pigs. It would not be expected to do so in mice and rabbits unless the deleterious effect of enterotoxin was not confined to the alimentary tract; the Ent⁺ *Salmonella* strains did not dilate the ligated intestine of these two animal species. In calves and pigs the chain of events beginning with the entry of enteropathogenic *Escherichia coli* into the small intestine and ending with the outpouring of fluid into this organ is undoubtedly complex. It would be unlikely to occur as a result of salmonellas entering the alimentary tract purely because they were able to produce *E. coli* enterotoxin. The pathogenesis of infections with enteropathogenic *E. coli* and with *Salmonella* are markedly different. For example, in the *E. coli* infections the bacteria remain localized in the alimentary tract whereas in *Salmonella* infections they invade the body. In *E. coli* infections the mucous membrane of the small intestine exhibits no obvious histological changes (Smith & Jones, 1963) whereas in *Salmonella* infections it is severely inflamed (Smith & Jones,

1967); in the calves used in the present study, extensive necrosis of the mucous membrane of the small intestine had occurred within a few days of their infection with salmonellas. It is therefore not surprising that the diarrhoea in the calves and pigs infected with Ent⁺ *Salmonella* strains was no more severe than in calves and pigs infected with Ent⁻ strains; the mechanism which gives rise to *E. coli* diarrhoea probably could not operate.

It appears that enterotoxin production is yet another characteristic that can be transmitted from bacterium to bacterium. The number of such characteristics continues to grow. Although much remains to be discovered, the point has now been reached at which the transmissible plasmid must be regarded as a normal method of inheritance in some bacterial species, rather than as a rare biological accident.

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