Plasmid and chromosomal related toxin polymorphism of *Escherichia coli* serogroup O138; plasmid transfer and co-integration with pRP4

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Summary. DNA–DNA hybridisation was used to examine 89 isolates of *Escherichia coli* serogroup O138 for toxin gene content and location. Sixty-six isolates encoded toxin genes; 29 were STpa STpb VT2, 24 were VT2, four were STpa STpb, three were STpb VT2, three were STpb VT2 LT and one was STpa. None were K88-, K99- or F41-positive. The VT toxin gene was chromosomally located in all VT⁺ isolates tested whereas STpa, STpb and LT were plasmid borne. In co-transfer experiments with pRP4 as the mobilising plasmid, VT2 genes were not transferred to recipient *E. coli* K12 whereas STpa, STpb and LT genes were. Two VT2-positive isolates inhibited pRP4 transfer to K12 by a factor of 10⁶ and rare transconjugants harboured a co-integrate plasmid comprising pRP4 (54kb) and a cryptic host plasmid (145 kb). In a K12 background, the co-integrate had transfer and incompatibility properties of pRP4 whereas in both progenitor *E. coli* O138 backgrounds the co-integrate was transfer inhibited and in one case resolved to give original plasmids.

Introduction

Escherichia coli serotype O138:K81 has long been associated with oedema disease and post-weaning diarrhoea in pigs (Sojka *et al.*, 1957). This serotype has subsequently been shown to be present in pigs in many countries (Sojka, 1965). Oedema disease is a toxæmic disease (Sojka, 1965) and many of the classical *E. coli* strains associated with the disease produce a toxin that causes a cytotoxic effect in Vero cells (Smith *et al.*, 1983). Likewise Dobrescu (1983) and Linggood and Thompson (1987) examined a number of oedema disease-producing strains of *E. coli*; although VT was detected, other *E. coli* enterotoxins were uncommon. During the development of gene probes for the detection of *E. coli* toxins in this laboratory, it was observed that some strains of *E. coli* O138:K81 produced a wide range of toxins (Woodward *et al.*, in press). Transmissible plasmids encoding combinations of enterotoxin and adhesin genes have been described (Smith *et al.*, 1985) and it seems likely that toxin polymorphism might be attributed to possession of such plasmids. However, Gannon *et al.* (1988) were unable to demonstrate transmissibility of genes encoding verocytotoxin from verocytotoxigenic

E. coli isolates from pigs. The porcine verocytotoxin, also referred to as Shiga-like toxin II variant (SLT-IIV) by Marques *et al.* (1987) is antigenically distinct from the human verocytotoxins, VT1 and VT2 (Law, 1988; Smith and Scotland, 1988; Karmali, 1989) but the gene encoding porcine verocytotoxin shows significant DNA sequence homology with VT2 (Smith *et al.*, 1988). The location of SLT–IIV genes is unknown whereas VT1 and VT2 genes were isolated from temperate bacteriophages in *E. coli* of serogroup O157 isolated from man (Willshaw *et al.*, 1985, 1987). In this paper, we report our initial studies aimed at understanding the genetic basis of toxin polymorphism in *E. coli* serogroup O138, which may assist in elucidation of the aetiology of the economically important disease of post-weaning diarrhoea.

Materials and methods

Bacterial strains and plasmids

E. coli strains isolated from pigs with intestinal infection during the period 1986–1989 were stored on Dorset egg slopes (PM5; Oxoid) at room temperature. All isolates were serotyped and tested for the presence of adhesins by standard methods based on those of Sojka (1965). Those belonging to serogroup O138 were plated

on blood agar and individual colonies were used in DNA-DNA hybridisation or plasmid profiling. *E. coli* K12 strains were 5K F⁻ *thr leu lacY tonA21 hsdRS14 rpsL thi1* (Colson *et al.*, 1965), J62 F⁻ *lac pro his trp*, and J62-1 F⁻ *lac pro his trp nal*. Plasmids were pRP4 Ap Km Tc *incP* 54kb and pR751 Tp *incP* 45kb (National Collection of Type Cultures, plasmid section, Central Public Health Laboratory, Colindale, London).

Plasmid profiles

Plasmids were extracted by the alkaline lysis method of Birnboim and Doly (1979) and lysates were subjected to agarose 0.7% gel electrophoresis with TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.6) at 3 V/cm at 4°C. Gels were stained in TAE buffer containing ethidium bromide 0.5 µg/ml for 40 min and destained in distilled water for 10 min before visualisation under UV illumination.

Colicin sensitivity testing

The overlay and mitomycin C-induction of colicin techniques of Pugsley and Oudega (1987) were used.

Conjugation

Conjugation experiments were performed as described previously (Woodward *et al.*, 1989a).

DNA-DNA hybridisation

Derivation of gene probes. The LT probe was a 750-bp *Hind*III fragment derived from pEWD299 (Dallas *et al.*, 1979). The STpa probe had a 157-bp *Hinf*I fragment derived from pRIT10036, formerly pCLS2 (Lathe *et al.*, 1980; Moseley *et al.*, 1980). The STpb probe was a 460-bp *Hinf*I fragment derived from the smaller of two *Hind*III fragments of pRAS, formerly pCHL6 (Lee *et al.*, 1983). The VT1 probe was a 530-bp *Hind*III/*Acc*I fragment derived from pNTP705 (Willshaw *et al.*, 1985). The VT2 probe was a 660-bp *Eco*RV/*Hinc*II fragment derived from pNTP707 (Willshaw *et al.*, 1987). The K99 probe was a 1250-bp *Bgl*II/*Sma*I fragment derived from pFK99 (de Graaf *et al.*, 1984). The K88 probe was a 1700-bp *Eco*RI fragment derived from pFM205 (Moseley *et al.*, 1986). The F41 probe was a 660 bp *Hinc*II/*Sal*I fragment derived from pDGA9 (Anderson and Moseley, 1988).

Probe preparation. Probe fragments were purified by electro-elution of DNA-containing bands cut from agarose (if more than 500 bp) or polyacrylamide (if less than 500 bp) gels of restriction enzyme digests. After electro-elution, probe DNA fragments were washed successively in phenol, phenol:chloroform 1:1 and chloroform (Maniatis *et al.*, 1982) and concentrated by precipitation under 2.5 volumes of absolute ethanol at -70°C after addition of sodium acetate to 150 mM. Probe DNA was radiolabelled to about 1×10^9 cpm/µg specific

activity with α^{32} PdCTP by random hexanucleotide primer DNA synthesis (Multiprime, Amersham) as described by Feinberg and Vogelstein (1983).

Colony lysis. Cultures for testing were grown overnight in Brain-Heart Infusion broth at 37°C with agitation. From each culture, 5 µl were transferred to the surface of a nylon membrane (Hybond-N, Amersham) overlaid on a dried MacConkey agar plate. Inoculated filters were incubated at 37°C for 4 h. Colonies from conjugation experiments were transferred to nylon membranes by replica plating. Colonies were lysed by laying filters colony side up sequentially on blotting paper saturated with SDS 5% for 5 min, 0.5 M NaOH, 1.5 M NaCl for 15 min and 1.5 M NaCl, 1.0 M Tris-HCl, pH7 for two 7-min periods based on the technique of Mainil *et al.* (1985). Air dried filters were wrapped in Saran wrap and exposed to UV irradiation (302 nm) by inversion over a transilluminator (Ultra Violet Product Inc.) for 4 min to fix the DNA.

Hybridisation. When probing for LT, STpa or STpb genes, filters were prehybridised for at least 1 h at 65°C in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH7), 5 × Denhardt's solution (1 × Denhardt's is bovine serum albumin (Pentax fraction V) 0.02% w/v, Ficoll Mr 400 000 0.02% w/v, polyvinylpyrrolidone Mr 360 000 0.02% and SDS 0.5% w/v containing sonicated heat denatured salmon sperm DNA (Sigma) 20 µg/ml. When probing for VT1, VT2, K99, K88 or F41 genes, filters were prewashed at 42°C for 2 h in 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA and SDS 0.1% followed by prehybridisation for 4 h at 42°C in formamide 50% v/v, 5 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA), 5 × Denhardt's solution and SDS 0.1% w/v containing sonicated heat denatured salmon sperm DNA 100 µg/ml. Radiolabelled probes were denatured at 100°C for 2 min then immediately placed in ice and added to the filters (10 ng of probe DNA per 10 cm × 10 cm filter) in fresh hybridisation solution. The probes were allowed to hybridise for 16 h; hybridisations with LT, STpa and STpb were performed at 65°C and those with VT1, VT2, K99, K88 and F41 at 42°C. Post-hybridisation stringency washes for all probes were in 2 × SSC, SDS 0.1% at room temperature (4 × 10 min), 1 × SSC, SDS 0.1% at 68°C (2 h) and 0.2 × SSC, SDS 0.1% at 68°C (1 h). After air drying, filters were exposed to X-ray film (Fuji RX) with intensifying screens (Cronex) at -70°C.

Results

Toxin profile of *E. coli* O138 strains

Porcine *E. coli* isolates belonging to serogroup O138:K81, which had been stored in the *E. coli* reference laboratory at the Central Veterinary Laboratory since 1986, were tested for the presence of toxin genes by DNA hybridisation. Of the 89 isolates, two were isolated from 1-month-old piglets with systemic disease, one was from an adult pig

with diarrhoea and the remainder were from post-weaning piglets aged 4–7 weeks. Sixty-six isolates were shown to harbour at least one toxin gene; 29 were STpa, STpb, VT2 positive, 24 were VT2 positive, four were STpa, STpb positive, three were STpb, VT2 positive, three were STpb, VT2, LT positive and one was STpa positive. The two isolates from systemic disease were STpa STpb positive and STpa STpb VT2 positive respectively. None of the isolates produced K99, F41 or K88 fimbriae as determined by either agglutination tests or by hybridisation with adhesin specific gene probes.

Location of toxin genes

The location of toxin genes on plasmids and their transmissibility *in vitro* has been well documented (Smith *et al.*, 1985). However, the question remains as to why *E. coli* serogroup O138 demonstrates considerable diversity with respect to toxin type and the combinations of toxins produced. To locate the toxin genes, Southern transfers of plasmid profiles of 24 isolates representing each of the toxin combination genotypes were tested by hybridisation with each toxin gene probe. The results are given in table I. All isolates harboured at least one

plasmid with as many as seven in two isolates. The method for plasmid extraction was that of Birnboim and Doly (1979) and a considerable chromosomal smear was always present as demonstrated by visualisation of ethidium bromide staining of agarose gel electrophoresis separated cell lysates. Without exception, the VT2 probe hybridised with the chromosomal smear of all 15 VT2⁺ isolates tested. Probes specific for STpa, STpb and LT hybridised with plasmid DNA but only with a single plasmid in any one isolate. Within any one toxin genotype, common toxin bearing plasmids were detected such as a 112-kb STpb LT, plasmid in the three STpb LT VT2 isolates tested, a 120-kb STpa STb plasmid in three of five and a 125-kb STpa STpb plasmid in two of five STpa STpb VT2 isolates and a 112-kb STpa STpb plasmid in three of four STpa STpb isolates.

Transmissibility of toxin genes

To test for transmissibility of toxin genes, filter mating experiments were set up. However, direct selection for transfer of toxin genes was not possible. Therefore, to demonstrate that gene transfer had occurred in any filter mating with an O138 donor and K12 recipient, co-transfer experiments were

Table I. Plasmid profiles of toxigenic *E. coli* of serogroup O138 and location of toxin genes

Isolate no.	Toxin genes encoded	Number of plasmids	Approximate size of plasmids (kb)	Location of toxin gene
M470-86	VT2	3	165, 90, 22	chr (VT2)
M471-86	VT2	3	165, 90, 22	chr (VT2)
M140-87	VT2	4	145, 120, 6.9	chr (VT2)
M634-86	VT2	6	120, 115, 90	chr (VT2)
M160-87	VT2	6	145, 105, 6.9	chr (VT2)
M213-86	STpa	3	120, 90, 60	120 (STpa)
M426-86	STpb	4	120, 90, 57, 6	90 (STpb)
M269-87	STpb	3	150, 90, 72	90 (STpb)
M 25-86	STpa STpb VT2	2	165, 120	chr (VT2) 120 (STpa STpb)
M 49-86	STpa STpb VT2	3	165, 125, 9	chr (VT2) 125 (STpa STpb)
M 91-86	STpa STpb VT2	2	165, 120	chr (VT2) 120 (STpa STpb)
M101-86	STpa STpb VT2	5	165, 120, 51, 5.7, 4.5	chr (VT2) 120 (STpa STpb)
M500-88	STpa STpb VT2	4	170, 125, 69, 6	chr (VT2) 125 (STpa STpb)
M243-87	STpb VT2	5	160, 128, 82, 6.2, 6	chr (VT2) 82 (STpb)
M627-86	STpb VT2	7	160, 115, 110, 90, 67, 64, 5.4	chr (VT2) 90 (STpb)
M 92-88	STpa STpb	4	120, 112, 52, 30	112 (STpa STpb)
M108-88	STpa STpb	1	112	112 (STpa STpb)
M458-88	STpa STpb	7	160, 135, 112, 60, 7.5, 7, 6.3	112 (STpa STpb)
M136-86	STpa STpb	5	150, 142, 45, 5.3, 4.5	142 (STpa STpb)
M576-86	STpb LT VT2	2	112, 36	chr (VT2) 112 (STpb LT)
M419-88	STpb LT VT2	4	157, 112, 30, 5.9	chr (VT2) 112 (STpb LT)
M421-88	STpb LT VT2	4	157, 112, 30, 5.9	chr (VT2) 112 (STpb LT)

Location of genes was determined by DNA hybridisation with Southern blot of plasmid profiles; chr, chromosomal DNA; size (in kb) indicates the plasmid that gave the hybridising signal.

performed in which the donor carried a promiscuous selectable plasmid. Transconjugants were then probed for the presence of toxin genes. The *incP* plasmid, pRP4, was introduced by filter mating into representative antibiotic-sensitive O138 isolates. The donor was strain J62pRP4 and selection was made on glucose minimal medium containing kanamycin 40 µg/ml. The frequencies of transfer were satisfactory, being between 2×10^{-2} and 6×10^{-5} /donor compared with 10^{-1} /donor in a K12 to K12 control mating. Derivatives of isolates M140-87(VT2), M160-87(VT2), M245-87(STpa), M269-87(STpb), M25-86 (STpa STpb VT2), M243-87 (STpb VT2), M458-88 (STpa STpb) and M576-86 (STpb LT VT2) bearing pRP4 did not grow when plated on glucose minimal medium containing streptomycin 500 µg/ml and in filter matings of the form M140-87pRP4 $\times$ *E. coli* K12 strain 5K *thr leu thi rpsL* selection was made on glucose minimal medium supplemented with thiamine 20 µg/ml, threonine 50 µg/ml and leucine 50 µg/ml and to which kanamycin 40 µg/ml and streptomycin 500 µg/ml were added as counterselective agents. The frequencies of pRP4 transfer are given in table II. With the exceptions of isolates M140-87 and M160-87, pRP4 transfer ranged from 10^{-3} to 10^{-4} although this was markedly less than for K12 to K12 transfer. Nylon membranes were overlaid on selection plates and the colonies were removed and the cells were lysed. These colony blots were allowed to hybridise with the relevant toxin gene probe (table II). The apparent frequency of transfer of a toxin gene was calculated from the number of hybridisation-positive colonies against the dilution

Fig. 1. Autoradiograph showing the hybridisation of the STpa-specific gene probe with transconjugant colonies which had been replicated and lysed on a nylon membrane. The mating was of isolate M245-87 pRP4 (STpa⁺) with strain 5K, with selection for transfer of pRP4 on counterselective media containing kanamycin spread with undiluted mating mixture to give confluent growth; the resultant colonies were transferred to a nylon membrane by replica plating and lysed with 0.5 M NaOH before hybridisation.

factor from which the membrane was prepared. An example of an autoradiograph from which data was obtained is shown in fig. 1. The gene encoding VT2 was not transferred in any experiment. Where the donor harboured a multiple toxin plasmid (M25-86, M458-88, M576-86), the frequencies of transfer of each toxin from any one donor were identical and the same transconjugant colonies hybridised,

Table II. Plasmid and toxin gene transfer from *E. coli* of serogroup O138

pRP4 harbouring O138 derivatives and toxin genotype	Frequency of pRP4 transfer into <i>E. coli</i> strain	Frequency of transfer of toxin genes as determined by DNA hybridisation of pRP4 transconjugants			
	K12 5K	VT2	STpa	STpb	LT
M140-87 (VT2)	5×10^{-8} *	$< 10^{-9}$	...	...	...
M160-87 (VT2)	8×10^{-8} *	$< 10^{-9}$	...	...	...
M245-87 (STpa)	4×10^{-4}	...	7×10^{-7}	...	...
M269-87 (STpb)	6×10^{-4}	...	...	8×10^{-7}	...
M25-86 (STpa STpb VT2)	3×10^{-4}	$< 10^{-9}$	6×10^{-7}	6×10^{-7}	...
M243-87 (STpb VT2)	6×10^{-3}	$< 10^{-9}$	...	6×10^{-6}	...
M458-88 (STpa STpb)	6×10^{-3}	...	4×10^{-7}	4×10^{-7}	...
M576-86 (STpb LT VT2)	8×10^{-4}	$< 10^{-9}$	...	1×10^{-8}	1×10^{-8}
<i>E. coli</i> K2 J62	1.1×10^{-1}	...	...	...	...

* Only 63 transconjugants from three separate mating experiments were obtained and none hybridised with the VT2 toxin gene probe.

suggesting transfer *en bloc*. The frequencies of toxin transfer were (in general) about 10^{-3} -fold less than of pRP4, which suggests that the toxin genes were not carried by highly promiscuous plasmids. The inability to pick transconjugant colonies from confluent replica plates and the destructive nature of the hybridisation protocol precluded the isolation of transconjugants to determine whether toxin genes had been mobilised with pRP4 as entire autonomous plasmids, or as co-integrates or had transposed on to pRP4.

The effect of colicins upon plasmid transfer

The transfer of pRP4 from *E. coli* K12 into O138 isolates and the subsequent transfer of pRP4 from O138 transconjugants back into *E. coli* K12 may have been hindered by killing of *E. coli* K12 by colicins produced by O138 isolates. Indeed, plasmid profiles showed that 12 of the 24 O138 isolates harboured small plasmids (4–8 kb in size) which may encode colicins (Pugsley and Oudega, 1987). The overlay technique and mitomycin C induced cell lysate technique demonstrated that *E. coli* K12 strain 5K, used as the recipient in mating experiments, was killed by M25–86, M140–87, M160–87, M243–87 and M458–88. The question arose as to whether the killing effect, whether or not the result of colicin or bacteriophage activity, was sufficient to explain the dramatic decrease in pRP4 transfer from strains M140–87 and M160–87 to *E. coli* K12

strain 5K. Repeat mating experiments were set up with strains M140–87 pRP4 and M160–87 pRP4 as donors with strain 5K as recipient, and selection was made for strain 5K alone on minimal medium supplemented with streptomycin. The number of strain 5K surviving was about 4×10^7 /filter compared with 2×10^8 /filter in a control experiment with strain J62pRP4 as donor and strain 5K as recipient. Twenty-four surviving colonies of strain 5K from each of the experiments with pRP4-positive isolates M140–87, M160–87 and J62pRP4 as donor were screened for plasmid content; those with strains M140–87 and M160–87 as donor had received a 6.9-kb plasmid whereas no other plasmids were detected in 5K recipients from any donor. It is possible that the 6.9-kb plasmid conferred colicin (or bacteriophage immunity) resulting in survival of the cells of strain 5K.

Stable cointegrate formation with pRP4

The very low frequency of transfer of pRP4 from isolates M140–87 and M160–87 to strain K12 may have been caused by some active mechanism, perhaps fertility inhibition. Plasmid profiles of isolates M140–87, M160–87, pRP4 derivatives and 5KpRP4 transconjugants were prepared (fig. 2, lanes 6–12). Isolate M140–87 harboured two large plasmids of about 145 kb and 120 kb (lane 12) and isolate M160–87 harboured two large plasmids of about 145 kb and 105 kb (lane 11). Both isolates

Fig. 2. Cell lysates of *E. coli* O138 strains and derivatives run through agarose 0.7% and visualised under transillumination after ethidium bromide staining: M140–87 (lane 12); M160–87 (lane 11); J62 pRP4 Ap^r Tc^r Km^r (lane 10); M140–87 pRP4 (lane 9); M160–87 pRP4 (lane 8); 5K Ap^r Tc^r Km^r, transconjugant designated 140.2, derived from a mating with M140–87 pRP4 as donor (lane 7); 5K Ap^r Tc^r Km^r transconjugant, designated 160.2, derived from a mating with M160–87 pRP4 as donor (lane 6); *E. coli* K12 plasmid mol. wt reference strain harbouring plasmids of 170 kb and 56 kb (lane 5); M160–87 Ap^r Tc^r Km^r transconjugants derived from a mating with 160.2 as donor (lanes 3 and 4); M140–87 Ap^r Tc^r Km^r transconjugants derived from a mating with 140.2 as donor (lanes 1 and 2). Chromosomal DNA is indicated by chr.

M140-87 and M160-87 harboured a 6.9-kb putative colicin plasmid (not shown). Derivatives of isolates M140-87 and M160-87 harbouring pRP4 had a discrete plasmid band of 54 kb and a plasmid band whose migration suggested a plasmid of about 200 kb (lanes 9 and 8 respectively). A total of 63 transconjugants from three experiments, 13 from matings with strain M160-87pRP4 as donor and 50 from matings with strain M140-87pRP4 as donor, were shown to be resistant to kanamycin (Km) 40 µg/ml, tetracycline (Tc) 40 µg/ml, ampicillin (Ap) 100 µg/ml and streptomycin 500 µg/ml, to require thiamine, threonine and leucine for growth on minimal medium and to be sensitive to phage Pl, thus confirming them to be derivatives of strain 5K. All 63 transconjugants harboured the 6.9-kb plasmid (not shown) and a presumptive pRP4 co-integrate form of about 200 kb (lanes 6 and 7). The 200-kb plasmids were shown to be stable by plasmid profiling individual colonies from six successive platings of transconjugants on blood agar supplemented with kanamycin.

Properties of the pRP4 co-integrate

Two 5K transconjugants harbouring the 200-kb putative pRP4 co-integrate, designated 140.2 and 160.2, derived from matings with donors M140-87pRP4 and M160-87pRP4 respectively, were

analysed further. To test whether the low frequency of transfer of pRP4 from isolates M140-87 and M160-87 to K12 was a property of the co-integrate plasmid, mating experiments were performed in which transconjugants 140.2 and 160.2 were donors and strain J62 was the recipient. Selection was made for transfer of each of the antibiotic resistances encoded by pRP4, separately. The frequency of transfer was about 1.4×10^{-1} from both donors. Resistance to kanamycin, tetracycline and ampicillin were transferred *en bloc* as shown by replica plating and all J62 transconjugants tested harboured two plasmids, one of 200 kb and another of 6.9 kb.

As a co-integrate form, the question arose as to which incompatibility functions of the two plasmids, pRP4 and the 145-kb cryptic plasmid would be dominant in any particular host. In an incompatibility experiment, transconjugants 140.2 and 160.2 were donors in matings with isolates M140-87 and M160-87, respectively, as recipients. It was thought that the co-integrate would enter the recipient and, if selection was made for pRP4-encoded antibiotic resistances and the co-integrate remained stable, the incompatible cryptic plasmid would be lost. With 140.2 as donor and M140-87 as recipient, the frequency of transfer was 5.8×10^{-4} . Twenty-four kanamycin resistant transconjugants were shown by plasmid profiling to have

Fig. 3. Cell lysates of transconjugant 140.2 and derivatives run through agarose 0.7% and visualised under UV transillumination after ethidium bromide staining: 140.2 Ap^r Tc^r Km^r (lane 8); J62.1 pR751 Tp^r showing ccc and oc forms (lane 7); four 140.2 Tp^r Ap^s Tc^s Km^s transconjugants derived from a mating with J62.1 pR751 Tp^r as donor with selection for transfer of Tp^r (pR751) into 140.2 (lanes 9-12); and six 140.2 Tp^r Ap^r Tc^s Km^s transconjugants derived from a mating with J62.1 pR751 Tp^r as donor with selection for transfer of Tp^r (pR751) and retention of TnA (Ap^r) in 140.2 (lanes 1-6). Chromosomal DNA is indicated by chr.

the 200-kb co-integrate plasmid, the 120-kb cryptic plasmid and the 6.9-kb plasmid (not shown) but not the 145-kb cryptic plasmid (fig. 2, lanes 3 and 4). With transconjugant 160.2 as donor and isolate M160.87 as recipient, the frequency of transfer was 4×10^{-4} . Twenty-four kanamycin resistant transconjugants contained plasmids of 145 kb, 105 kb, 54 kb (pRP4) and 6.9 kb (not shown) with a plasmid band corresponding to 200 kb (fig. 2, lanes 1 and 2) indicating resolution of the 200-kb co-integrate plasmid in some but not all transconjugants.

In another incompatibility experiment, transconjugants 140.2 and 160.2 were used as recipients and the *incP* plasmid, pR751, which encodes trimethoprim (Tp) resistance, was introduced by filter mating with strain J62.1 pR751 as donor. Selections were made on trimethoprim 10 µg/ml alone or in combination with either, tetracycline 40 µg/ml or ampicillin 50 µg/ml. In a control experiment, strains J62.1 pR751 and 5K were mated with selection for transfer of pR751 into 5K on trimethoprim 10 µg/ml; the frequency of transfer was 5×10^{-2} . With transconjugant 140.2 as recipient, the frequency with which trimethoprim resistant transconjugants were obtained was 5.8×10^{-4} . Trimethoprim and ampicillin resistance transconjugants were obtained at a frequency of 7.3×10^{-5} . No trimethoprim- and tetracycline-resistant transconjugants were obtained. Fig. 3 shows plasmid profiles (excluding the 6.9-kb plasmid) of donor J62.1 pR751 (lane 7) in both ccc and oc forms, recipient 140.2 (lane 8), four trimethoprim-resistant transconjugants (lanes 9–12) and six trimethoprim- and ampicillin-resistant transconjugants (lanes 1–6). It would appear that in a K12 background, incompatibility between pR751 and pRP4 in co-integrate form was demonstrated. When selecting for pR751 on trimethoprim alone, pRP4 excised from the co-integrate and was lost leaving the 145-kb cryptic plasmid. However, with selection for both trimethoprim and ampicillin, excision of pRP4 from the co-integrate was imprecise as shown by the varying sizes of the residual cryptic plasmid presumably tagged with at least the *bla* gene of pRP4.

Derivatives of isolates M140–87 and M160–87 which had received the co-integrate (pRP4: 145-kb cryptic) plasmid by filter mating were used as donors in matings with *E. coli* K12 strain 5K as recipient to test whether transfer functions had been restored. In these experiments, eight randomly chosen donors from each of isolates M140–87 and M160–87 were used with selection being made separately for ampicillin, tetracycline or kanamycin. The frequencies of transfer were in the range 8×10^{-7} to 7×10^{-8} . Transfers of resistances were

shown, by replica plating, to be *en bloc* and plasmid profiles of 5K transconjugants revealed one plasmid of 200 kb and another of 6.9 kb.

Discussion

E. coli of serogroup O138 showed considerable toxin gene polymorphism. Whilst the verocytotoxin is a significant factor in causing post-weaning diarrhoea and oedema disease (Nielsen, 1981; Marques *et al.*, 1987), the contribution to the development of disease made by enterotoxins is unclear. The lack of known adhesins in these enterotoxigenic isolates suggests an inability to cause disease by the same mechanism as typical enterotoxigenic *E. coli* (ETEC) unless these isolates harbour a novel adherence mechanism. Whether O138 isolates lacking toxin genes altogether were pathogenic is not known, although Matthewson *et al.* (1985) have demonstrated a mild diarrhoeal disease caused by adherent non-toxigenic strains. It is tempting to speculate that toxin polymorphism is, at least in part, a reflection of the range of disease syndromes associated with *E. coli* O138 in pigs.

The toxin polymorphism displayed by serogroup O138 may be attributable to a dynamic interchange of toxin genes by plasmids, bacteriophages and transposons. However, the VT2 gene was chromosomal; whether the VT2 gene was integrated as part of a temperate bacteriophage as in O157:H7:VT2⁺ isolates (Willshaw *et al.*, 1987) is not known. The VT2 gene was not mobilised by pRP4, a finding which confirms that of Gannon *et al.* (1988). Here may be evidence for derivation of VT⁺ and VT⁻ clones of serogroup O138.

Toxin-encoding plasmids have been known for some time (Smith *et al.*, 1985) and may have been acquired recently as entire entities by individual O138 strains by conjugation. Alternatively, such plasmids may have been produced by co-integration of two or more toxin-gene bearing plasmids upon residence in O138 strains. The possession of transmissible toxin plasmids suggests that serogroup O138 is a potential source for the dissemination of toxin genes to other bacteria.

The mechanism for repression of pRP4 transfer by two O138 isolates is unclear. However, repression was mediated through the cytoplasm and not as a result of co-integration affecting genetic function of pRP4 because transfer was still dramatically reduced even when the plasmids were resolved in an O138 background. It is intriguing that escape from repression of transfer occurred at a very low frequency resulting in the production of a stable co-integrate in the recipient. In a K12

background, the co-integrate plasmid behaved as plasmid RP4 with respect to mobility and incompatibility which suggests that the repression mechanism was only operative in an O138 background. Whether the repression mechanism was plasmid encoded, but not expressed in the co-integrate form in K12, or chromosomal is unknown. If the repression of transfer function was chromosomal then perhaps the common concept of plasmid promiscuity (Farrar, 1983; Thompson, 1986) needs to be reconsidered because the two isolates described here may have evolved a plasmid capture mechanism. It might explain plasmid and plasmid-borne toxin gene polymorphism in *E. coli* serogroup O138. Other organisms such as *S. typhimurium* DT204C are known to readily accumulate plasmids (Wray *et al.*, 1987) whereas others such as *S. dublin* and *S. enteritidis* generally carry a single plasmid (Woodward *et al.*, 1989a, b). Of course, plasmid transfer and carriage depends upon ecological factors (Corpet, 1986) and plasmid polymorphism may be a reflection of the environment of *E. coli* O138 rather than their innate genetic makeup.

Clues to the mechanism of plasmid co-integration were gained by incompatibility experiments. Plasmid pRP4 encodes TnA (Tn3), a prototype for class II transposons (Kleckner, 1981) which are known to generate co-integrates as normal intermediates in transposition (Arthur and Sherratt, 1979). Ben-

nett *et al.* (1986) described replicon fusion by one-ended transposition in which the co-integrate was not resolved, presumably associated with loss of the resolvase function, as would appear to be the case in this study. However, upon incompatible challenge of the co-integrate with R751, pRP4-encoded functions were lost *en bloc* indicating that resolvase was able to excise pRP4 from the co-integrate. Also, when the co-integrate was introduced into an O138 background, plasmid resolution was observed. Here was evidence that a resolvase capability, whether or not TnA-encoded, functioned in K12 and O138 backgrounds. Interestingly, in the incompatibility challenge experiments it was demonstrated that excision of pRP4 from the co-integrate was often imprecise, as evidenced by the retention of ampicillin resistance. Perhaps the active sites for excision recombination events were in or around TnA, which encodes ampicillin resistance, which might be further evidence for intimate involvement of TnA in the initial co-integration. Notwithstanding a probable role for TnA in co-integration, the effect of a cryptic plasmid in an O138 background upon pRP4 behaviour was significant. This indicates a role of such cryptic plasmids for plasmid capture and co-integration *in vivo*; it is possible that multiple toxigenic plasmids have been formed by such co-integration events.

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