Evidence for Related Virulence Sequences in Plasmids of *Salmonella dublin* and *Salmonella typhimurium*

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Transposon-insertion mutants were prepared from virulent field isolates of *Salmonella dublin* and *Salmonella typhimurium*. Detailed restriction-enzyme mapping of the single sites of TnA insertion in two mutants (M51 and M173) of *S. dublin* that showed diminished virulence in a mouse assay indicated that these sites were about 5 kbp apart on the approximately 70 kbp plasmid harboured by the isolate. A Tn10-insertion mutant (M242) of *S. typhimurium* that showed diminished virulence was also identified. A single copy of Tn10 was inserted into the approximately 90 kbp plasmid harboured by this isolate. Hybridization studies indicated that homology existed between the region encompassing the sites of TnA insertion in M51 and M173 and that encompassing the site of Tn10 insertion in M242. Restriction mapping indicated that the two regions were very similar and could even be identical and, if so, the Tn10 insertion in M242 could be mapped to a point 1.5 kbp from the TnA insertion in M51 and 6.5 kbp from that in M173. It appeared that the maximal extent of the putative similarity/identity was between 13 and 23 kbp. It is proposed that this stretch of high homology could represent a virulence sequence that has been conserved during the evolutionary divergence of the two *Salmonella* serotypes.

INTRODUCTION

Plasmids play a part in the virulence of a number of pathogenic bacteria. For example, plasmids carry genes encoding toxin production, iron sequestration, adhesiveness and serum resistance (Levy *et al.*, 1981).

In the last few years it has been shown by experiments involving curing or conjugational transfer that plasmids play an important role in mediating the virulence of enteroinvasive bacteria such as *Shigella* and *Salmonella*. Thus, Sansonetti *et al.* (1981, 1982) showed that the presence of a 185 or a 215 kbp plasmid is necessary to enable *Shigella sonnei* or *Shigella flexneri*, respectively, to produce keratoconjunctivitis in the guinea-pig. Similarly, Jones *et al.* (1982) found that the ability of virulent *Salmonella typhimurium* strains to adhere to and invade HeLa cells, and to kill mice following oral administration, depends on the presence of a 92 kbp plasmid. Terakado *et al.* (1983) reported that loss of a 75 kbp plasmid present in numerous virulent *Salmonella dublin* isolates leads to diminished ability to kill mice following peritoneal inoculation.

Relatively little is known about the nature of the plasmid genes that are involved in virulence expression in these enteroinvasive bacteria, or about the proteins that these genes encode. However, Hale *et al.* (1983) have been able to associate loss of virulence in *Shigella flexneri* with a large deletion of unspecified size in the 215 kbp plasmid, and consequent failure to synthesize seven plasmid-encoded outer-membrane polypeptides in minicell experiments.

The present study describes the isolation of two transposon-insertion mutants of *Salmonella dublin* and one of *Salmonella typhimurium* that show diminished virulence in a mouse assay.

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline.

Investigations with these three mutants have led to the identification of regions of the approximately 70 and 90 kbp plasmids present in the *S. dublin* and *S. typhimurium* isolates, respectively, that must play an important role in virulence. There appears to be substantial homology between these regions in the two serotypes, suggesting the presence of conserved virulence sequences. Some preliminary findings have been published (Baird & Manning, 1984; Manning *et al.*, 1984).

METHODS

Bacterial strains and plasmid vectors. These are listed in Table 1. Bacteria were stored in 12% (v/v) glycerol at -70°C as described by Silhavy *et al.* (1984).

Preparation of mutants. Transposon-insertion mutants of *S. dublin* 2229 and *S. typhimurium* 1275 were prepared by random insertion of either TnA (Ap^r), originating on plasmid pMR5 (RP1ts) (Robinson *et al.*, 1980), or of Tn10 (Tc^r), present in a suicide derivative of phage P22 (Davis *et al.*, 1980).

To prepare TnA-insertion mutants, a copy of plasmid pMR5 was transferred to the wild-type *Salmonella* isolate from *Escherichia coli* K12(pMR5) by conjugation (Miller, 1972). Transconjugants were identified by plating out the conjugation mix on modified brilliant green agar (Oxoid CM329) containing Ap ($500\text{ }\mu\text{g ml}^{-1}$), Km ($30\text{ }\mu\text{g ml}^{-1}$) and Tc ($20\text{ }\mu\text{g ml}^{-1}$). Procedures were carried out at 30°C . Descendants of these transconjugants that were Ap^r but Km^s and Tc^s after 18 h growth at 42°C on two separate occasions were taken to be TnA-insertion mutants. Tn10-insertion mutants were prepared as described for *S. typhimurium* LT2 by Davis *et al.* (1980). In this method, the suicide phage is prepared by mixing mutant, tail-deficient P22::Tn10, obtained by inducing the lysogenic strain *S. typhimurium* NK337, with head-deficient P22, obtained by growing P22-503 in a non-suppressing host (see Table 1). Tc^r descendants of wild-type *Salmonella* isolates infected with the suicide P22::Tn10 were taken to be Tn10-insertion mutants. The identity and status of all *Salmonella* mutants were validated using qualitative slide agglutination reactions for the common serotype-specific O and H antigens (Difco and Wellcome Diagnostics) (Kauffmann, 1972), and, where necessary, sugar fermentations (API Carbohydrate System) and biochemical tests for *Enterobacteriaceae* (Difco) (see also Buchanan & Gibbons, 1974).

Assessment of virulence. Virulence of the transposon-insertion mutants was assessed by comparing their ability to kill mice with that of the wild-type parent, following oral inoculation. The mice were 6–8-week-old C57BL/COM females, boxed individually and given Clarke's Rat and Mouse pellets and water *ad libitum*.

Bacterial strains to be tested were grown for 18 h at 37°C in static conditions in L broth alone (wild-type strains), in L broth containing $500\text{ }\mu\text{g Ap ml}^{-1}$ (TnA-insertion mutants), or in L broth containing $20\text{ }\mu\text{g Tc ml}^{-1}$ (Tn10-insertion mutants). (L broth contains 10 g tryptone, 5 g yeast extract and 5g NaCl l^{-1} ; pH 7.5.) The bacteria were washed at least once in isotonic saline (0.9%, w/v, NaCl) and 0.1 ml volumes of appropriate dilutions in isotonic saline of these washed cells were then inoculated into each of 4–16 mice. In all tests, at least one control group of mice was inoculated with the wild-type parent and another with isotonic saline. The mice were monitored for up to 14 d, and deaths recorded. Most deaths occurred between 5 and 10 d following inoculation.

In all, about 500 mutant strains were screened in the present study.

Preparation of plasmid DNA. Crude plasmid DNA was prepared by the method of Silhavy *et al.* (1984), using lysozyme and NaOH/SDS. The DNA was then purified by centrifugation to equilibrium in a CsCl/ethidium bromide gradient over 36 h at 15°C (Maniatis *et al.*, 1982). After removal of the ethidium bromide by butanol extraction (Davis *et al.*, 1980), the DNA was precipitated with ethanol and finally dissolved in 10 mM-Tris, 1 mM-EDTA, pH 8.0.

Restriction-enzyme digests. These were carried out as described by Maniatis *et al.* (1982).

Preparation of total DNA. Total (i.e. plasmid plus chromosomal) DNA was prepared essentially by the method described by Maniatis *et al.* (1982) for eukaryotic DNA, except that the bacteria were resuspended in 50 mM-glucose, 10 mM-EDTA, 25 mM-Tris/HCl, pH 8.0, containing 5 mg lysozyme ml^{-1} , and kept at 0°C for 30 min before being treated with 0.5% Sarkosyl, 0.5 M-EDTA, pH 8.0, containing 100 μg proteinase K ml^{-1} (Maniatis *et al.*, 1982).

Electrophoresis. Horizontal electrophoresis was carried out in 0.6% (w/v) agarose gels using Tris/acetate buffer (40 mM-Tris, 20 mM-acetic acid, 2 mM-EDTA, pH 8.1; Davis *et al.*, 1980). DNA was usually electrophoresed for about 15 h at 1.5 V cm^{-1} at room temperature.

Hybridization. DNA fragments in agarose gels were transferred to nitrocellulose filters by Southern blotting, and then hybridized to ^{32}P -labelled, nick-translated plasmid probes. The procedures employed were as described by Maniatis *et al.* (1982).

Cloning. Various restriction-digest fragments of the *S. dublin* and *S. typhimurium* wild-type and mutant plasmids were cloned for restriction mapping and hybridization studies. A given plasmid and the chosen vector, i.e. pACYC184, pAT153 or pUC12 (Table 1), were digested with an appropriate restriction endonuclease and the resultant fragments separated by electrophoresis. Following localization, gel pieces containing the bands of cut vector and plasmid fragment were excised, placed in dialysis bags and subjected to electroelution for 3–4 h at

Table 1. *Strains and vectors used*

Strain/vector	Properties	Source*	Reference
<i>Salmonella</i> strains			
<i>S. dublin</i> 2229		Field outbreak of bovine salmonellosis	
<i>S. typhimurium</i> 1275		Field outbreak of bovine salmonellosis	
<i>S. typhimurium</i> NK337	<i>supE</i> [P22 (c2 ts 12 am 13 am int Tn10)]	D. Botstein ¹	Davis <i>et al.</i> (1980)
<i>S. typhimurium</i> TR248		D. Botstein ¹	Davis <i>et al.</i> (1980)
<i>S. typhimurium</i> TR251	<i>supD</i>	D. Botstein ¹	Davis <i>et al.</i> (1980)
<i>S. dublin</i> M51	TnA-insertion mutants of <i>S. dublin</i> 2229 exhibiting diminished virulence in mice	Prepared by the authors	
<i>S. dublin</i> M173			
<i>S. dublin</i> M114	TnA-insertion mutant of <i>S. dublin</i> 2229 that retained full virulence	Prepared by the authors	
<i>S. typhimurium</i> M242	Tn10-insertion mutant of <i>S. typhimurium</i> 1275 exhibiting diminished virulence in mice	Prepared by the authors	
<i>E. coli</i> strains			
<i>E. coli</i> K12(pMR5)	Harbours plasmid pMR5, i.e. RPlts; this plasmid contains TnA	H. R. Smith ²	Robinson <i>et al.</i> (1980)
<i>E. coli</i> HB101	<i>hsdS recA</i>	J. G. Williams ³	Boyer & Roulland-Dussoix (1969)
Phage P22-503	<i>c1</i> 12 am 13 am	D. Botstein ¹	Davis <i>et al.</i> (1980)
Vectors			
pACYC184	4 kbp; Cm ^r Tc ^r	M. Kehoe ⁴	Chang & Cohen (1978)
pAT153	3.6 kbp; Ap ^r Tc ^r	J. G. Williams ³	Twigg & Sherratt (1980)
pUC12 (same cloning site as M13 mp10)	2.8 kbp; Ap ^r	Bethesda Research Laboratories, Paisley, UK	Messing & Vieira (1982)

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5 V cm⁻¹. The electrocluted DNA was then extracted with phenol/chloroform, precipitated with ethanol and finally dissolved in 10 mM-Tris, 1 mM-EDTA, pH 8.0. Following incubation of the vector with alkaline phosphatase, appropriate vector and plasmid fragments were mixed and incubated in the presence of ligation buffer and T4 DNA ligase at 4 °C for 18 h (Maniatis *et al.*, 1982). The ligated DNA was extracted with phenol/chloroform and ethanol-precipitated. Finally, the DNA, dissolved in 10 mM-Tris, 1 mM-EDTA, pH 8.0, was transformed into *E. coli* HB101 (Maniatis *et al.*, 1982). Identity of clones was confirmed by restriction digestion and hybridization.

RESULTS

Mutants exhibiting diminished virulence

Three mutants were identified that exhibited diminished virulence. Of these, two, designated M51 and M173, were TnA-insertion mutants of *S. dublin* 2229, while the other, designated M242, was a Tn10-insertion mutant of *S. typhimurium* 1275. Table 2 records the results obtained in separate virulence assays with these three mutants. The assays indicated that the virulence of M51 and M173 was some 10⁴–10⁵-fold less than that of the parent *S. dublin* 2229, i.e. that 10⁴–10⁵ times as many c.f.u. of either mutant were required to achieve the same lethality as a given

Table 2. *Comparison of ability of mutants M51, M173 and M242, and their wild-type parents, to kill mice*

The inoculum volume was 0.1 ml. Numbers of c.f.u. per 0.1 ml of undiluted inoculum were, respectively: for wild-type and M51, 7.3 and 6.1×10^7 ; for wild-type and M173, 4.8 and 5.0×10^7 ; for wild-type and M242, 5.2 and 3.9×10^7 . For further details, see Methods.

Dilution inoculated	Mice killed/mice inoculated					
	Expt 1		Expt 2		Expt 3	
	<i>S. dublin</i> 2229	M51	<i>S. dublin</i> 2229	M173	<i>S. typhimurium</i> 1275	M242
Undiluted	—	9/16	4/4	0/4	—	—
10^{-1}	—	0/16	4/4	1/4	—	—
10^{-2}	—	0/16	4/4	0/4	—	2/7
10^{-3}	—	0/16	3/4	0/4	6/7	3/7
10^{-4}	7/8	—	3/4	0/4	5/7	0/7
10^{-5}	5/8	—	2/4	0/4	4/7	0/7
10^{-6}	—	—	—	—	1/7	—

inoculum of 2229. The virulence of M242 was some 10^2 – 10^3 -fold less than that of the parent *S. typhimurium* 1275. Assays of the virulence of M51, M173 and M242 were repeated on at least two further occasions, using a similar experimental design. These repeat assays gave similar results (not shown) to those recorded in Table 2.

General properties of mutants M51, M173 and M242

No differences were observed between the mutants and their respective parents in colony morphology, qualitative agglutination reactions, biochemical properties or resistance to rabbit serum (Taylor *et al.*, 1972). The *in vitro* growth rates of *S. dublin* M51 and M173 in L broth at 37 °C were the same as that of the parent wild-type. By contrast, the *in vitro* growth rate of *S. typhimurium* M242, as with other, virulent, Tn10-insertion mutants, was up to 50% less than that of the parent.

Plasmid content

Electrophoretic analysis, using plasmids of known size as markers (not shown), indicated that *S. dublin* 2229 harboured one plasmid species of 69 kbp, while *S. typhimurium* harboured at least two species, one of 88 kbp and the other of about 3 kbp. The single plasmid species present in *S. dublin* 2229 and the larger species present in *S. typhimurium* 1275 were of similar size to those plasmids which have previously been implicated in the virulence of these serotypes (Jones *et al.*, 1982; Terakado *et al.*, 1983). Electrophoretic analysis also gave values of 71 and 75 kbp for the plasmids present in *S. dublin* M51 and M173, respectively, and of 96 kbp for the larger plasmid present in *S. typhimurium* M242. These mutant plasmids were, therefore, larger than the corresponding wild-type plasmids, indicating that transposon insertion into the plasmids had probably occurred in all three mutants.

S. typhimurium M242 was cured of its 96 kbp plasmid, containing Tn10, by selecting for tetracycline sensitivity (Bochner *et al.*, 1980). The virulence of the cured derivative was about 10^2 -fold less than that of M242, i.e. 10^4 – 10^5 -fold less than that of 1275 (data not shown).

Mapping of transposon location

S. dublin M51 and M173. Restriction digest experiments with enzymes for which there is no recognition site in TnA (*Eco*RI, *Hind*III, *Sac*I, *Sal*I, *Xho*I) indicated that both M51 and M173 contained a single TnA insertion in the 70 kbp plasmid, and that the sites of insertion in the two mutants were not more than 10 kbp apart. In M51, TnA insertion had taken place with the deletion of some 2–3 kbp of DNA (Fig. 1a). More detailed mapping, including the use of enzymes for which there are asymmetrically positioned sites in TnA (*Cla*I, *Bam*HI), indicated

Fig. 1 (a) Partial restriction map of the *S. dublin* 2229 plasmid showing approximate insertion sites of TnA in M51 and M173 (black wedges). The plasmid is drawn to scale on the assumption that its size is 70 kbp. The marked *EcoRI* and *SacI* sites are missing in M51.

(b) Restriction map of approx. 15 kbp stretch of the 70 kbp plasmid of *S. dublin* 2229 showing the sites of insertion of TnA in M51, M114 and M173. The thick line indicates the extent of the deletion in M51. The arrows indicate the orientation of TnA in each mutant. Restriction enzyme abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Nde*I; S, *Sal*I; Sc, *Sac*I; X, *Xho*I.

that the TnA copies in M51 and M173 were located 5 kbp apart and were inserted in opposite orientations (Fig. 1b). It was also possible to map the position of a neighbouring TnA insertion found in a third mutant, designated M114, that retained full virulence in the mouse assay. This latter insertion was about 4 kbp from that in M173, on the opposite side from that in M51 (Fig. 1b).

S. typhimurium M242. Restriction digest experiments with enzymes for which there is no restriction site in Tn10 (*Sac*I, *Sal*I, *Xho*I) indicated that in M242 a single copy of Tn10 was inserted into the 90 kbp plasmid present in 1275. Mapping with these enzymes, and with others that cut Tn10 asymmetrically (*Eco*RI, *Hind*III), permitted the construction of the restriction map illustrated in Fig. 2.

Apparent similarity of transposon insertion sites. Restriction digest profiles of the 70 and 90 kbp plasmids of *S. dublin* 2229 and *S. typhimurium* 1275, respectively, were clearly different for any given restriction enzyme. However, the restriction maps for the regions of transposon insertion in *S. dublin* M51 and M173, on the one hand (Fig. 1b), and in *S. typhimurium* M242 on the other

Fig. 2. Restriction map of approx. 35 kbp stretch of the 90 kbp plasmid of *S. typhimurium* 1275 showing the site of insertion in M242 of Tn10, whose orientation is indicated by the arrow. Restriction enzyme abbreviations as in Fig. 1 (b). The zig-zag lines indicate the probable maximum limits of inter-serotype homology (see also text).

(Fig. 2), were markedly similar. In fact, the distribution of sites in the *XhoI/SalI* fragments containing the transposon insertions in M51 and M242 appeared to be identical, suggesting that these fragments must at least share substantial homology.

The existence of homology in the regions of transposon insertion in the two plasmids had been confirmed at an early stage of the work when it had been observed that the *SalI* fragment encompassing the sites of TnA insertion in *S. dublin* M51 and M173 hybridized to the *SalI*, *XhoI* and *PstI* fragments of the *S. typhimurium* M242 plasmid that contained the Tn10 insertion (Fig. 3a, b). Subsequently, it was found that the similar *XhoI/SalI* fragments from the two serotypes hybridized strongly. More detailed studies indicated that the homology extended leftwards beyond the left-hand *SacI* site of the *S. dublin* 2229 plasmid (Fig. 1b) but ceased before the *SalI* site was reached. The probable maximum limits of the inter-serotype homology in this region of the 1275 plasmid are indicated in Fig. 2. Whereas the left-hand limit must be approximately correct, the right-hand limit merely indicates the end of known similarity in restriction-site distribution.

Possible presence of chromosomal transposon insertions

The theoretical possibility existed that further transposon copies could have been inserted into the chromosomal DNA of the three mutants. To investigate this possibility, autoradiograph bands that appeared when a clone containing the relevant transposon was hybridized to restriction digests of mutant total DNA were compared with those appearing when the clone was hybridized to parallel digests of plasmid DNA from the same strain. For all three mutants, the autoradiograph bands produced by hybridization of the appropriate clone to total DNA corresponded to those produced when the clone was hybridized to plasmid DNA. It could therefore be concluded that, in the case of each mutant, the clones were hybridizing to plasmid DNA contained within the total DNA, but were failing to hybridize to chromosomal DNA. Evidently, none of the three mutants contained a copy of the inserted transposon in its chromosomal DNA.

DISCUSSION

Transposon insertion interrupts the continuity of a given DNA sequence and may therefore be expected to impair or abolish its genetic function. The fact that the single transposon insertions in the three mutants that exhibited diminished virulence were all into the large plasmids harboured by *S. dublin* and *S. typhimurium* indicates that these plasmids play an important role in virulence expression. In permitting this conclusion, the present work corroborates the previous findings of other workers, made on the basis of curing and conjugation experiments (Jones *et al.*, 1982; Terakado *et al.*, 1983).

It was of interest that a single transposon insertion was sufficient to cause such a large decrease in virulence in *S. dublin* M51 and M173. In each of these mutants, the insertion must have destroyed the whole of the contribution of the plasmid to virulence (as assayed in the

Fig. 3(a) Comparison of restriction digest profiles of plasmid fractions obtained from *S. typhimurium* 1275 and Tn10-insertion mutant M242. Contents of gel tracks reading from the left: (1), (3), (5), (7), (9) and (11), *S. typhimurium* 1275 cut with *Eco*RI, *Hind*III, *Sal*I, *Xho*I, *Pst*I and (*Pst*I + *Xho*I), respectively; (2), (4), (6), (8), (10) and (12), mutant M242 cut with *Eco*RI, *Hind*III, *Sal*I, *Xho*I, *Pst*I and (*Pst*I + *Xho*I), respectively. The two outer tracks (faint) are of λ cut with *Hind*III.

(b) Autoradiograph of hybridization of a 32 P-labelled clone, containing the *S. dublin* 2229 *Sal*I fragment that encompasses the sites of transposon insertion in M51 and M173, to *S. typhimurium* 1275 and M242 plasmid digests. The clone was hybridized to DNA from the gel illustrated in (a). The fragments hybridizing in each track are as follows (lettering from origin - see a): (1) B, C, F and H; (2) A, C and D; (3) B, C, F and H; (4) B, C, F and H; (5) A; (6) A; (7) C; (8) C; (9) C, G and H; (10) A, D and H; (11) C, G and H; (12) A, D and H.

animal model used in this study), since curing M173 of its plasmid failed to diminish virulence any further (E. J. Manning, G. D. Baird & P. Jones, unpublished). This was not the case with *S. typhimurium* M242, since curing this strain of the larger plasmid did lead to a further decrease in virulence (see above). The cured derivatives of *S. dublin* 2229 and *S. typhimurium* 1275, and also M51 and M173, were still able to kill mice if administered in sufficient quantity. This residual virulence must have been chromosomally encoded.

The fact that the transposon insertions in *S. dublin* M51 and M173 were only 5 kbp apart suggests that the insertions could have taken place into a discrete sequence that encoded the virulence factors. This possibility is supported by further observations. Firstly, while the region encompassing the sites of TnA insertion in *S. dublin* M51 and M173 appeared, on the basis of restriction sites, to be very similar to the region into which Tn10 is inserted in *S. typhimurium* M242, flanking sequences to the left and right of this region were clearly dissimilar. The virulence-impairing insertions in the two serotypes appear, therefore, to have been into what may be closely related sequences of limited length. Secondly, while the TnA insertion in *S. dublin* M114 was only 4 kbp distant from that in M173, it was on the edge of the region of putative identity, and also failed to diminish virulence. The possibility therefore exists that the regions of apparent homology represent a virulence-encoding sequence that has been preserved during the evolutionary divergence of the two serotypes.

The finding of homology between sequences of *S. dublin* and *S. typhimurium* plasmids is in accord with the recent work of Popoff *et al.* (1984), who observed homology between these plasmids and also those of other *Salmonella* serotypes. Hale *et al.* (1983) earlier observed homology in the large (185–215 kbp) plasmids present in *Shigella flexneri*, *Shigella sonnei* and enteroinvasive *Escherichia coli*. These latter authors also provided evidence for the existence of a discrete virulence-encoding region in the *S. flexneri* plasmid, since a mutant carrying a deletion of unspecified size in this plasmid was avirulent.

There are at least three possible explanations for the fact that transposon insertions that were 5 kbp apart in the *S. dublin* plasmid should both apparently destroy the total virulence capacity of the plasmid. These are (i) that several plasmid genes play an equally indispensable role in virulence expression, (ii) that at least one of the transposons was inserted into a regulatory sequence, and (iii) that at least one of the transposons was exerting polarity effects on distant sequences. Genetic and sequencing analyses will be required to resolve this question. It is only possible to speculate at this stage on reasons why the Tn10 insertion in *S. typhimurium* M242 did not reduce virulence further. One possibility is that this was due to the relative importance of interrupted sequences for virulence expression. Another is that the *S. typhimurium* plasmid could contain further virulence genes, located elsewhere, that are not possessed by the *S. dublin* plasmid.

The finding that only one transposon copy was inserted into each mutant was not unexpected, in the case of M242, at least. The transposition frequency of Tn10 from a phage suicide vector is about 2×10^{-6} (Foster *et al.*, 1981), and this normally ensures that only one copy of this transposon is present in each mutant clone (Davis *et al.*, 1980). The frequency of transposition of TnA is higher, i.e. about 1×10^{-4} and 3×10^{-8} per MDal of recipient plasmid or chromosomal DNA, respectively (Kretschmer & Cohen, 1977) and the possibility of multiple transposon insertions in a given mutant might, therefore, be correspondingly greater. Nevertheless, this would be unlikely to result in multiple insertions into the same replicon, owing to immunity effects (Robinson *et al.*, 1977).

It might have been expected that some mutants showing diminished virulence would have been found that had chromosomal transposon insertions. Thus, it is known that mutations in certain chromosomal metabolic loci, e.g. those in the *galE* locus (Germanier & Fürer, 1975) and in the *aroA* locus (Hoiseth & Stocker, 1981) result in loss of virulence in *Salmonella*. That no such insertions were found in the present study may simply have been due to the relatively small numbers of mutants screened.

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REFERENCES

- BAIRD, G. D. & MANNING, E. J. (1984). Molecular evidence for plasmid involvement in the virulence of *Salmonella dublin*. In *Priority Aspects of Salmonellosis Research*, pp. 147–153. Edited by H. E. Larsen. Brussels: Commission of the European Communities (Publication EUR 9197EN).
- BOCHNER, B. R., HUANG, H. C., SCHIEVEN, G. L. & AMES, B. N. (1980). Positive selection for loss of tetracycline resistance. *Journal of Bacteriology* **143**, 926–933.
- BOYER, H. W. & ROULLAND-DUSSOIX, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *Journal of Molecular Biology* **41**, 459–472.
- BUCHANAN, R. E. & GIBBONS, N. E. (1974). *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore: Williams & Wilkins.
- CHANG, A. C. Y. & COHEN, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *Journal of Bacteriology* **134**, 1141–1156.
- DAVIS, R. W., BOTSTEIN, D. & ROTH, J. R. (1980). *Advanced Bacterial Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- FOSTER, T. J., DAVIS, M. A., ROBERTS, D. E., TAKESHITA, K. & KLECKNER, N. (1981). Genetic organization of transposon Tn10. *Cell* **23**, 201–213.
- GERMANIER, R. & FÜRER, A. (1975). Isolation and characterization of *galE* mutant Ty21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. *Journal of Infectious Diseases* **131**, 553–558.
- HALE, T. L., SANSONETTI, P. J., SCHAD, P. A., AUSTIN, S. & FORMAL, S. B. (1983). Characterization of virulence plasmids and plasmid-associated outer membrane proteins in *Shigella flexneri*, *Shigella sonnei* and *Escherichia coli*. *Infection and Immunity* **40**, 340–350.
- HOISETH, S. K. & STOCKER, B. A. D. (1981). Aromatic-dependent *S. typhimurium* are non-virulent and effective as live vaccines. *Nature, London* **291**, 238–239.
- JONES, G. W., RABERT, D. K., ŠVINARICH, D. M. & WHITFIELD, H. J. (1982). Association of adhesive, invasive, and virulent phenotypes of *Salmonella typhimurium* with autonomous 60-megadalton plasmids. *Infection and Immunity* **38**, 476–486.
- KAUFFMANN, S. (1972). *Serological Diagnosis of Salmonella Species*. Copenhagen: Munksgaard.
- KRETSCHMER, P. J. & COHEN, S. N. (1977). Selected translocation of plasmid genes: frequency and regional specificity of translocation of the Tn3 element. *Journal of Bacteriology* **130**, 888–899.
- LEVY, S. B., CLOWES, R. C. & KOENIG, E. L. (1981). *Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids*. New York and London: Plenum Press.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- MANNING, E. J., BAIRD, G. D. & JONES, P. W. (1984). Possible plasmid involvement in the virulence of *Salmonella dublin*. *Biochemical Society Transactions* **12**, 847–848.
- MESSING, J. & VIEIRA, J. (1982). A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene* **19**, 269–276.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- POPOFF, M. Y., MIRAS, I., COYNAULT, C., LASSELIN, C. & PARDON, P. (1984). Molecular relationships between virulence plasmids of *Salmonella* serotypes typhimurium and dublin and large plasmids of other *Salmonella* serotypes. *Annales de microbiologie* **135A**, 389–398.
- ROBINSON, M. K., BENNETT, P. M. & RICHMOND, M. H. (1977). Inhibition of TnA translocation by TnA. *Journal of Bacteriology* **129**, 407–414.
- ROBINSON, M. K., BENNETT, P. M., FALKOW, S. & DODD, H. M. (1980). Isolation of a temperature-sensitive derivative of RP1. *Plasmid* **3**, 343–347.
- SANSONETTI, P. J., KOPECKO, D. J. & FORMAL, S. B. (1981). *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. *Infection and Immunity* **34**, 75–83.
- SANSONETTI, P. J., KOPECKO, D. J. & FORMAL, S. B. (1982). Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infection and Immunity* **35**, 852–860.
- SILHAVY, T. J., BERMAN, M. L. & ENQUIST, L. W. (1984). *Experiments with Gene Fusions*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- TAYLOR, P. W., ROBERTS, A. P. & GOWER, P. L. (1972). Evaluation of a technique for the estimation of serum bactericidal activity against Gram-negative organisms. *Medical Laboratory Technology* **29**, 272–279.
- TERAKADO, N., SEKIZAKI, T., HASHIMOTO, K. & NAITOH, S. (1983). Correlation between the presence of a fifty-megadalton plasmid in *Salmonella dublin* and virulence for mice. *Infection and Immunity* **41**, 443–444.
- TWIGG, A. J. & SHERRATT, D. (1980). Trans-complementable copy number mutants of plasmid ColE1. *Nature, London* **283**, 216–218.