The virulence plasmid of *Salmonella dublin*: detailed restriction map and analysis by transposon mutagenesis

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A detailed restriction map of the virulence plasmid of *Salmonella dublin* has been determined and used for comparison with the virulence plasmid from *S. typhimurium*. Two regions were identified which appeared to be similar based on blotting and restriction data. One, of about 22 kb, encompassed the virulence region; the other, of about 8 kb, was outside it. The locations of 259 transposon insertions on the *S. dublin* plasmid were determined and related to their effect on virulence. One gene involved in virulence but outside the essential virulence region was shown to affect citrate metabolism.

Introduction

The carriage of plasmids by *Salmonella* species is important for virulence. Jones *et al.* (1982) showed that removal of the large cryptic plasmid from *S. typhimurium* led to a reduction in mouse virulence, and similar effects have been noted for *S. dublin*, *S. enteritidis*, *S. gallinarum*, *S. pullorum* and *S. choleraesuis* (Terakado *et al.*, 1983; Nakamura *et al.*, 1985; Barrow *et al.*, 1987; Barrow & Lovell, 1988; Kawahara *et al.*, 1988). It has been demonstrated that these large virulence plasmids are involved in systemic infection (Pardon *et al.*, 1986; Manning *et al.*, 1986) and do not affect the ability of *Salmonella* to cause enteric disease (Jones *et al.*, 1988).

The plasmids carried by different serotypes differ in their size and restriction digest pattern (Popoff *et al.*, 1984; Helmuth *et al.*, 1985). However, plasmids from different serotypes share substantial homology in a region shown to be involved in virulence (Baird *et al.*, 1985; Baird & Manning, 1984; Williamson *et al.*, 1988*a*; Woodward *et al.*, 1989). Transposon mutagenesis of the *S. dublin* and *S. typhimurium* plasmids identified some genes within this region which were involved in virulence (Baird *et al.*, 1985; Beninger *et al.*, 1988; Gulig & Curtiss, 1988; Rhen *et al.*, 1989). We and others have recently shown that a part of this region is sufficient to restore virulence to cured *S. dublin* and *S. typhimurium* isolates (Williamson *et al.*, 1988*b*; Beninger *et al.*, 1988; Gulig & Curtiss, 1988; Norel *et al.*, 1989*a*). Our work also demonstrated that regions linked to virulence lie

outside the essential region (Williamson *et al.*, 1988*b*).

The size of the plasmid regions involved in virulence remains unclear. This paper describes a comprehensive restriction map for the plasmid commonly found in *S. dublin*. The information was used to compare the plasmid with the *S. typhimurium* plasmid and to analyse a bank of 233 transposon-insertion mutants of *S. dublin*, each of which contained an insertion in the virulence plasmid. Several more mutants with insertions within or adjacent to the virulence region were also generated and analysed for virulence. One of these latter mutants, G19 (Williamson *et al.*, 1988*b*), identified a gene located 5 kb outside the essential virulence region, which was shown to be involved in virulence and the ability to grow on citrate.

Methods

Strains and culture conditions. The wild-type *Salmonella* strains and some of the transposon-insertion mutants have already been described (Baird *et al.*, 1985; Manning *et al.*, 1986; Williamson *et al.*, 1988*a, b*). Bacterial stocks were stored in 12% (v/v) glycerol at -70°C . Bacteria were routinely grown in Luria–Bertani medium at 37°C (Maniatis *et al.*, 1982).

Plasmid procedures. The rapid method of Ish-Horowitz & Burke (1981) was used with the following modifications, which were found to be necessary for digestion of plasmid DNA from salmonellae. The bacteria were lysed at 37°C for 10 min using 5 mg lysozyme ml^{-1} . After incubation with SDS/NaOH on ice for 5 min, the mixture was incubated with sodium acetate (3 M, pH 4.8) for 10 min on ice. The plasmid isolation procedures described by Baird *et al.* (1985) were also used.

DNA was electrophoresed in horizontal 0.6% agarose gels in Tris/acetate buffer (Maniatis *et al.*, 1982) and fragments were isolated either by electroelution (Maniatis *et al.*, 1982) or by absorption onto silica (Geneclean; Stratech Biotech). The restriction maps were

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constructed by a combination of single and double digests of the whole plasmid and of isolated restriction fragments. The size of fragments in the range 10–20 kb was assessed using the following molecular mass markers: λ DNA cut either with *NaeI* and *SalI* (0.5, 12.7, 15.2 and 20 kb), or with *KpnI* (1.5, 17.0 and 30 kb).

Southern blotting techniques were as described previously (Williamson *et al.*, 1988a). The probe was prepared as follows. A plasmid preparation from *S. typhimurium* 12/75 was digested with *XbaI*, which cuts the plasmid once (Baird *et al.*, 1985), and the digest was run on an agarose gel to separate the linear virulence plasmid from the small plasmid present in this isolate. The single large plasmid band was excised and the DNA was purified as above and radioactively labelled using a Multiprime kit (Amersham).

Transposon insertion mutants. Tn801 insertion mutants were prepared and their virulence was assessed as previously described (Baird *et al.*, 1985), except where stated in the text. The new transposon insertion mutants described here were labelled M501–M590.

Transduction. Antibiotic resistance was transduced using bacteriophage P22 (HT1Aint) as described by Davis *et al.* (1980).

Test for ability to utilize citrate. Approximately 10^8 saline-washed, exponentially growing bacteria were spread on minimal medium [K_2HPO_4 (7 g), KH_2PO_4 (3 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $(NH_4)_2SO_4$ (1 g), Noble-agar (Difco) (20 g), distilled water (1 litre) containing sodium citrate (5 g) and nicotinic acid (0.01 g)]. Strains able to utilize citrate grew as lawns after 48 h incubation at 37 °C, whereas strains unable to utilize citrate did not grow.

Assays for virulence. The virulence of wild-type and mutant strains of *Salmonella* for mice was assayed as described previously (Baird *et al.*, 1985) using both the oral and intraperitoneal routes.

Biochemical assays. The ability of salmonellae to utilize a variety of carbohydrates was assayed using Microbact 24E kits (LIP Ltd, Shipley, West Yorkshire, UK). LPS was analysed as described previously (Williamson *et al.*, 1988b).

Results

Restriction map of the plasmid from *S. dublin* 2229

The physical map of the 76 kb plasmid from *S. dublin* was determined for 13 enzymes (Fig. 1). The location of several of the sites around the virulence region was already known (Baird *et al.*, 1985). The sizes of the fragments generated by each enzyme were calculated using molecular mass standards accurate for the sizes being examined, as were the sizes of the fragments produced by each enzyme in combination with *XbaI*, *XhoI* or *BamHI*. The fragments produced by digestion with *XhoI* and *XbaI* and also the largest fragment produced by digestion with *EcoRI* were isolated from agarose gels, and subjected to single and double digests by the other enzymes. These procedures generated most of the data for the map. The relative orientations of the small *EcoRI* fragments were determined by mapping a bank of clones of the plasmid (Williamson, 1988). There was also evidence to suggest that there was a small fragment between *EcoRI* fragments C and D. Briefly,

mapping with *SacI* and *BamHI* showed that the transposon insertions in two mutants (M513 and M587) were each located close to the junction of *EcoRI* fragments C and D. However, in each case, the fragments from an *EcoRI* digestion were the same size as fragments from the parent plasmid except that there was a new band of about 5.3 kb. This suggested that transposon insertion in both instances had been into an intervening fragment of about 0.3 kb. It is possible that this putative intervening fragment was caused by an artefactual duplication linked to transposition. However, the orientation of insertion in M513 was opposite to that in M587 and it is therefore unlikely that a similar duplication could have been produced by two independent mutational events.

The correct orientation of sites that appeared very close to each other was checked by double digests of the whole plasmid or of isolated fragments. The orientations of *KpnI* fragments E and F and of *HindIII* fragments F and I were not determined; the orientation of the latter is taken from the data of Beninger *et al.* (1988). Three *AvrII* sites were identified, but these were not mapped accurately.

A more detailed map of the virulence region was constructed for six restriction enzymes not previously mapped (*AvrII*, *SmaI*, *EcoRV*, *PvuI*, *HpaI* and *NdeI*), using a clone of the 14 kb *SalI* fragment B in pACYC184 (Fig. 2). Sites were positioned with reference to the known restriction sites in the vector (Rose, 1988) and by using double digests.

Southern blots of the *S. dublin* plasmid cut with *SalI*, *EcoRI*, *SacI*, *HindIII*, *KpnI*, *ClaI*, *SfiI* and *SpeI* were probed with the complete virulence plasmid from *S. typhimurium*. The extent of hybridization was assessed for each fragment and interpreted to show the predicted homology between the plasmids (Fig. 3). The available restriction data on *S. typhimurium* (Baird *et al.*, 1985; A. J. Lax, unpublished) and sequence data around the position of the G19 insertion (G. D. Pullinger, unpublished) was used to define the extent of homology around the virulence region. For example, one end of this region could be defined by the absence of a *SpeI* site in the *S. typhimurium* plasmid and the difference in enzyme sites from this point rightwards (see Fig. 1). The other end could be defined by differences in sequence starting 700 bp to the right of the *SalI* site between fragments B and D in the *S. dublin* plasmid and proceeding leftwards. Overall the results indicated that the homology extended from map position 59 through 76.7/0 to approximately 5 kb. Restriction data previously obtained by Baird *et al.*, (1985 and unpublished) indicated that there was an extra sequence of about 2 kb in the *S. typhimurium* plasmid which was missing in the *S. dublin* plasmid at about map position 62 (Fig. 1). In addition to the homology

Fig. 1. Restriction map of the virulence plasmid from *S. dublin* isolate 2229. The following abbreviations are used: B, *BamHI*; C, *ClaI*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; N, *NotI*; Nh, *NheI*; S, *SacI*; Sc, *SacI*; Sf, *SfiI*; Sp, *SpeI*; X, *XhoI*; Xb, *XbaI*. The second line shows the position of Tn801 in mutants that have insertions in *SacI* fragments D, E or F and also mutations which generated large deletions. Mutants listed above the line were avirulent in mice; those below were virulent. The direction of the arrow indicates the orientation of the transposon from the *ClaI* to the *BamHI* sites. The corresponding position of Tn10 in mutant M242, transduced from *S. typhimurium* (Williamson *et al.*, 1988a), is indicated. Boxed regions (M113, M63, M30 and M51) indicate where transposition has caused a measurable deletion.

Fig. 2. Detailed restriction map of the 14 kb *SalI* fragment B from the virulence plasmid of *S. dublin*. Un-numbered arrowheads indicate sites of transposon insertion which did not affect virulence. Sites of insertion which did decrease virulence, i.e. G19, M51, M173 and M242 (site corresponding to that in the *S. typhimurium* plasmid), are also indicated. The positions of the defined virulence genes (see text) are also shown.

described above, the hybridization experiments indicated a second region of homology between about 38 and 46 kb (see Figs 1 and 3).

Plasmid mutants

The sites of transposon insertion in a bank of 144 virulent Tn801 insertion mutants of *S. dublin* (Baird *et al.*, 1985) were determined by means of *Sac*I digests. A further 89 fully virulent mutants were prepared and analysed in the same manner, and the results of both sets of mutants (prefixed M) are summarized in Table 1. The mutants all contained Tn801 inserted in the virulence plasmid. Transposition of Tn801 into the *S. dublin* virulence plasmid was non-random, as has been shown previously (Heffron *et al.*, 1975), and which may reflect the sequence specificity of Tn801 (Tu & Cohen, 1980).

Mutants with insertions within *SacI* fragments D, E or F were mapped accurately, using *ClaI*, *BamHI* and *BamHI/EcoRI* digests and are shown in Fig. 1. Several insertions mapped in identical positions and for simplicity only one example is shown. No insertions were found that mapped to fragment G (Table 1). Twelve mutants had deletions which extended from the site of insertion. The deletions in M30, M63 and M113 were mapped and are shown on Fig. 1. The mutants with a transposon insertion in *SacI* fragments D, E and F, and M30, M63 and M113, were re-tested for mouse virulence by both the oral and the intraperitoneal route and all were confirmed to be fully virulent (data not shown).

Further transposon insertions were targeted into *EcoRI* fragments C and E, using cassette mutagenesis. To achieve this, fragments C and E were cloned into pACYC184, and these constructs were then used to transform the cured *S. dublin* strain (M173c: Manning *et al.*, 1986). Mutagenesis was carried out as described in Methods and ampicillin-resistant *Escherichia coli* were screened by *EcoRI* digestion of the plasmids to locate the position of insertion. The fragment sizes expected for inserts in *EcoRI* fragment C were either 4 and 13 kb, or 8 and 9 kb, depending on whether Tn801 (5 kb) was in the insert (8 kb) or vector (4 kb). However, three bands were present in each of 18 mutants tested (Fig. 4). This implied that two different plasmid species, one containing and one without the transposon, were able to coexist in these strains.

Inserts containing a transposon were subsequently ligated into the *Eco*RI site of pUB307 (Bennett *et al.*, 1977), and transformed into the semi-rough *S. typhimurium* strain, LB5010 (Williamson *et al.*, 1988*b*). Transduction of the ampicillin resistance into wild-type *S. dublin*

Fig. 3. Analysis of homology between the virulence plasmids of *S. dublin* and *S. typhimurium*. Southern blots of restriction digests of the *S. dublin* plasmid were probed with labelled *S. typhimurium* plasmid. The extent of hybridization for each fragment was assessed in one of five categories: very strong (■), strong (▣), weak (▤), very weak (▥), no hybridization (□). It was not possible to determine which *SalI* fragment C hybridized, but the interpretation shown is based on the absence of hybridization to *HindIII* fragments D and F. The composite interpretation was based mainly on fragments which did not hybridize, and on available restriction data (see text).

Table 1. Location of the position of the *Tn801* insertion

The *SacI* fragment containing *Tn801* was identified for each mutant. Numbers in parentheses indicate mutants in which a deletion was found. The three mutants with deleted sites had all lost the *SacI* site between the A and B fragments.

<i>SacI</i> fragment	No. of mutants	Percentage of total
A	35	15
B	66 (5Δ)	28
C	99 (3Δ)	43
D	6 (1Δ)	3
E	3	1
F	21	9
G	0	0
Deleted sites	3	1
Total	233	100

2229 yielded mutants in which *Tn801* had been inserted into either *EcoRI* fragment C or E by homologous recombination.

Of the 26 mutants produced in this series, 25 contained *Tn801* in *EcoRI* fragment C (prefixed G) and one contained *Tn801* in *EcoRI* fragment E (designated E2). The locations of the insertions were determined (Fig. 1), and the mutants were tested for mouse virulence. One mutant, G19, which was briefly described by Williamson *et al.*, (1988*b*), was avirulent (see below).

We have designated the genes defined by the insertions which reduce virulence as *vag* (for virulence associated gene). Thus *vagA* is defined by the insertion in M242 (Pullinger *et al.*, 1989), *vagB* by the insertion in M173 (Manning *et al.*, 1986) and *vagC* by the insertion in G19 described here (Fig. 2).

Mutant G19

The avirulent mutant G19 was 10^5 -fold less virulent than the parental strain when inoculated by either the oral or the intraperitoneal route (results not shown). The restriction map of the plasmid from G19 (pG19) showed that it did not have gross deletions or other obvious differences from the parental strain. The strain was smooth with a normal LPS ladder (results not shown). pG19 was used to transform the cured strain (M173c). This strain was also smooth and avirulent.

The ability of the mutant G19 to utilize various sugars was compared to the wild-type and cured strain. The mutant lacked the ability to utilize citrate as a sole carbon source; its ability to grow on other carbohydrates (Microbact 24E) was identical to the wild-type. Although M173c could utilize citrate, introduction of pG19 led to the loss of this property, which suggested that the mutation in G19 interfered with chromosomal expression of citrate utilization.

Fig. 4. *EcoRI* restriction digest profiles of 18 ampicillin-resistant clones containing the *EcoRI* fragment C inserted into pACYC184. The lanes at each side are *HindIII*-digested λ DNA molecular mass markers. The mutants in lanes 1, 2, 9, 11, 13, 14, 15 and 17 have *Tn801* in the insert; the others have *Tn801* in the vector.

Discussion

We describe here a detailed restriction map of the virulence plasmid commonly found in *S. dublin* isolates. Beninger *et al.* (1988) published a map of this plasmid based on five enzymes, in which the position of 23 sites was determined. Our map, which gives the positions of 65 enzyme sites for 13 enzymes, is in agreement with theirs, and, having more sites, makes it possible to identify and clone parts of the plasmid more easily. In addition we have constructed a detailed map of the virulence region (six more enzymes) which will aid its genetic analysis.

The *S. dublin* map showed a clustering of restriction sites which might reflect an unusual base composition and which might have some evolutionary significance. There were relatively few restriction sites for the 13 enzymes in the half of the plasmid, from position 5 kb to 43 kb. Among the sites that were present those with AT-rich sequences predominated. Thus, *Cla*I and *Hind*III, the only enzymes which cut more than twice within this region, both have AT-rich recognition sequences. Enzymes with CTAG within their recognition sequence cut the plasmid only rarely, which might be predicted from the low frequency of this tetranucleotide in bacterial genomes (McClelland *et al.*, 1987). Three of these enzymes, *Xba*I, *Nhe*I and *Spe*I, each had one site, while the other, *Avr*II, had three. The high number (five) of *Sfi*I sites, which were clustered over a 25 kb stretch, was unexpected since this enzyme has an 8 bp recognition site. The *S. typhimurium* plasmid has seven *Sfi*I sites (A. J. Lax, unpublished observations).

We produced several Tn801 insertions which did not affect virulence, and which mapped close to the insertions in the avirulent mutants G19 and M173. This indicated that the loss of virulence in G19 and M173 was probably not due to polar effects. The insertions in G19 and M173 lie outside the essential virulence region (Williamson *et al.*, 1988b), and the target sequences for these insertions are therefore probably involved in controlling the expression of virulence (Williamson *et al.*, 1988b). There were several differences between G19 and M173 which suggested that loss of virulence was caused by different mechanisms, perhaps indicating that *vagB* and *vagC* interact with different target genes. Mutant G19 could be complemented by the virulence region on a low-copy-number vector, whereas M173 could not (Williamson *et al.*, 1988b). The G19, but not the M173 insertion, affected citrate metabolism, and G19 and M173 behaved differently in calf challenge experiments (Jones *et al.*, 1988).

Transposon mapping suggested that only part of the plasmid was required for virulence, since the avirulent mutations were clustered and 27 kb of the plasmid could

be deleted without affecting virulence (Fig. 1). This agreed with the identification of a common virulence region amongst *Salmonella* virulence plasmids (Williamson *et al.*, 1988a; Woodward *et al.*, 1989). This region has also been located by subcloning experiments (Williamson *et al.*, 1988b; Gulig & Curtiss, 1988; Norel *et al.*, 1989a). We also used the *S. dublin* map to define the similarity between the *S. dublin* and *S. typhimurium* plasmids in more detail. Approximately 22 kb around the virulence region was very similar. There was also one other region common to both the *S. dublin* and *S. typhimurium* plasmids, but this was unlikely to be directly involved in virulence since it could be deleted (M30) without affecting virulence.

Neither the total size of the virulence region nor the number of genes involved is known. Three different genes within this region in *S. typhimurium* have recently been sequenced (*vagA*: Pullinger *et al.*, 1989; *mkfA*: Norel *et al.*, 1989b; *mkaA*: Taira & Rhen, 1989) and there is either direct sequence evidence or protein expression data that these genes are also present in *S. dublin* (G. D. Pullinger & A. J. Lax, unpublished observations). Although several proteins are encoded by the virulence region (Gulig & Curtiss, 1988; Williamson, 1988; Norel *et al.*, 1989a), their function has not been defined. We have previously shown that 8 kb from this region was sufficient for expression of virulence when it was present in low copy number (Williamson *et al.*, 1988b). This suggested that some genes within it were involved in control. The sequence of one gene (*vagA*) shows significant homology with two bacterial genes, *metR* and *lysR*, which are known to be involved in regulation of gene expression (Pullinger *et al.*, 1989). Others have identified an essential virulence region using deletions in the wild-type plasmid (Michiels *et al.*, 1987; Beninger *et al.*, 1988) or vectors which had comparatively low copy number (Gulig & Curtiss, 1988). Norel *et al.* (1989a) restored full virulence to a cured *S. typhimurium* with a high-copy-number clone containing most of the 8 kb region. However, the relative contribution of the plasmid to the ability of a strain to cause systemic disease varies with different serotypes. *S. typhimurium* is less affected by the removal of its virulence plasmid than *S. dublin* or *S. enteritidis* (Williamson *et al.*, 1988a) and there are serotypes (e.g. *S. typhi*) which do not possess virulence plasmids but cause systemic disease. There is no homology between the virulence plasmids and *S. typhi* chromosomal DNA (C. M. Williamson & D. Maskell, unpublished observations), so there might be an alternative set of chromosomal genes which can perform a similar function. The demonstration that some plasmid genes are involved in control and apparently affect chromosomal expression suggests a possible alternative theory that the gene pro-

ducts which enable salmonellae to survive systemically are chromosomally encoded in all serotypes and that the role of the plasmid is to control chromosomal expression. Serotypes without virulence plasmids or for which the plasmid plays a less prominent role might have circumvented these control pathways. In any event, analysis of the plasmid-encoded functions should give some insight into the way in which salmonellae survive systemically.

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