The role of plasmid genes in the pathogenicity of *Salmonella dublin*

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Summary. The virulence (expressed as LD50 values) for mice of two mutant strains of *Salmonella dublin*, both containing TnA insertions in the resident plasmid, was reduced by 10^4 – 10^5 when infection was by the oral or intravenous or intraperitoneal route. When the plasmid was lost from one of the mutants no further decrease in virulence was observed. Results also suggested that plasmid genes are not involved in the ability of *S. dublin* to cross the gut wall.

Introduction

Plasmids play a role in the pathogenicity of *Salmonella typhimurium* (Jones *et al.*, 1982) and *S. dublin* (Terakado *et al.*, 1983). Jones and his colleagues also showed that a plasmid in *S. typhimurium* was essential for adhesion to, and invasion of, HeLa cells (Jones *et al.*, 1982). The role in pathogenicity of the *S. dublin* plasmid has not been identified.

This paper describes two mutant strains of *S. dublin* which have substantially lost virulence through a single transposon insertion in the resident plasmid (mol. wt 46×10^6). The derivation of these strains from the wild type and the plasmid DNA of the wild-type and mutant strains are described in detail by Baird and Manning (1984). In this study, a comparison has been made of the behaviour of the parent and mutant strains in virulence experiments in mice in the hope of identifying the role of the plasmid in pathogenicity. Some results have been published in preliminary form (Manning *et al.*, 1984).

Materials and methods

Bacterial strains.

S. dublin strain 2229 was smooth, aerogenic and virulent for mice and calves. It was isolated from an outbreak of calf enteritis in Britain in 1979. The mutants also used in this study (strains M51 and M173) were transposon-insertion mutants of strain 2229. In each there was a single insertion of transposon TnA in the serotype-specific plasmid (mol. wt 46×10^6); in the case of

strain M51, the transposition event resulted in the deletion of a segment of DNA of *c.* 3 kilobase-pairs (Baird and Manning, 1984). Strain 173/2 was a cured derivative of mutant M173 without the 46×10^6 -mol. wt plasmid (see below). *Escherichia coli* K12 strain 14R525, a plasmid-free, serum-sensitive laboratory strain was kindly supplied by Dr H. R. Smith (P.H.L.S., Colindale, London).

Curing of plasmid DNA

Mutant M173, whose plasmid contained TnA encoding ampicillin resistance, was grown overnight in Luria-Bertani (LB) broth containing the curing agent ethidium bromide $10 \mu\text{g/ml}$. To enrich for ampicillin sensitivity (indicating possible loss of the plasmid) the method of Rodriguez *et al.* (1976) was adopted. One ml of the overnight broth culture was subcultured into 100 ml of fresh LB broth and incubated at 37°C for 1 h. Ampicillin was added to a final concentration of $500 \mu\text{g/ml}$, and the culture incubated at 37°C for a further 45 min. Cycloserine, which in this situation kills only actively growing, ampicillin-resistant bacteria, was then added to a final concentration of $100 \mu\text{g/ml}$. After incubation at 37°C for another 2.5 h, $100\text{-}\mu\text{l}$ portions of undiluted culture and of a 1 in 10 dilution were spread on LB agar plates which were incubated overnight at 37°C . Each plate was then replica-plated on to LB agar plates containing ampicillin $500 \mu\text{g/ml}$ and ampicillin-sensitive colonies were sought. Only one ampicillin-sensitive (Amp^s) derivative was identified in a total of *c.* 25 000 colonies examined. This derivative, designated 173/2, was serologically and biochemically identical to *S. dublin* strain M173. Plasmid analysis (see below) showed that it lacked the plasmid of mol. wt 46×10^6 .

Viable counts

Bacterial counts were determined in inocula and in other samples by making serial ten-fold dilutions of the samples in saline and spreading 0.1 ml of appropriate dilutions on LB agar plates. The number of cfu/ml or /g was calculated.

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Virulence testing

The virulence of each bacterial strain was tested in C57 BL/COM mice, except in two experiments in which Lac/G/COM mice were used. Strains were grown in LB broth at 37°C for 16 h. Dilutions were made in saline and administered orally to groups of eight mice (0.1 ml/mouse). Deaths were monitored up to 14 days and LD50 values calculated by the method of Reed and Muench (1938). The number of organisms administered to each animal was calculated from viable counts of the inocula. In some experiments the challenge doses were injected into the mice by the intraperitoneal (ip) or intravenous (iv) (tail vein) route (0.1 ml/mouse).

Counts of bacteria in spleens

The appropriate dilution (determined from preliminary experiments) of an overnight broth culture of each test strain was given by mouth to groups of C57BL/COM mice (0.1 ml/mouse). At various times after administration, spleens, usually from groups of four mice, were removed, pooled and weighed. Saline (9 ml/g of tissue) was added and samples were homogenised in Griffith's tubes. Appropriate dilutions of the homogenates were spread on LB agar plates for the determination of viable counts.

Plasmid analysis

Plasmids were extracted by the method of Birnboim and Doly (1979), and analysed by electrophoresis in 0.6% agarose gels run horizontally at 100 V for 5 h, in a Tris-acetate buffer system (Tris 4.48 g, sodium acetate 0.68 g, EDTA 1.49 g, in 1 L, pH 7.8). Plasmid bands were visualised by immersing the gel in ethidium bromide 50 µg/ml for 1–16 h.

Serum resistance determination

Serum resistance was assayed by the method of Taylor *et al.* (1972). Overnight broth cultures of each test organism were subcultured into fresh LB broth (0.5 ml into 10 ml) and grown to an OD₆₅₀ of 0.5. A ten-fold dilution was then made to give a concentration of $c. 2 \times 10^7$ bacteria/ml, and 0.5-ml portions of this suspension were added to various dilutions (in saline) of rabbit or bovine serum, to give a final assay volume of 2.5 ml. Samples were incubated at 37°C for 90 min. Appropriate dilutions of each sample were prepared and viable counts determined at times 0 and 90 min.

Haemagglutinin assays

Mannose-resistant and mannose-sensitive haemagglutinins were assayed by the microhaemagglutination method of Jones and Rutter (1974).

Results

LD50 values after oral administration

The oral LD50 value of strain 2229 was 10^4 – 10^5 less than that of strains M173 (table I; see also Baird and Manning, 1984) and M51 (table I). The virulence of the cured derivative (173/2) of strain M173 (fig. 1) was low in mice, similar to that of strain M173 itself (table I).

Invasiveness of wild-type and mutant strains

The invasiveness of the *S. dublin* strains, expressed as the ability of the bacteria to cross the gut wall and accumulate in various tissues after oral administration, was assessed because several recent reports have described virulence plasmids that confer invasive properties upon *S. typhimurium* (Jones *et al.*, 1982) and *Shigella* spp. (Hale *et al.*, 1983). For a given dose, the accumulation of strain M51 in spleen was lower than that of the parent strain 2229 (fig. 2A). After administration of a lethal dose of strain 2229, counts in spleen tissue reached 10^7 – 10^8 cfu/spleen by the time of death (day 4) (fig. 2A). After a dose of $c. 1$ LD50 of strain M51, bacteria accumulated to high counts in the spleen by day 7, but then counts dropped substantially (fig. 2B). This can probably be explained by the fact that each sample was a pool of four spleens and approximately half of the animals given this dose would be expected to die, therefore each pooled sample (up to day 10) would contain two spleens from mice that would have died and two from mice that would have survived. However no mice died after day 10, therefore all spleens taken from day 10 onwards would have been from mice that survived the challenge, and would have lower bacterial counts in

Table I. Virulence of *S. dublin* strain 2229, two mutant strains (M51 and M173) and a cured derivative (173/2)

Mouse strain	Route of inoculation	Log ₁₀ LD50* of strain			
		2229	M51	M173	173/2
C57BL/COM	oral	2.7	7.7	NT	NT
C57BL/COM	oral	2.8	NT	7.1	NT
C57BL/COM	oral	2.9	6.9	8.2	7.8
Lac/G/COM	oral	2.8	7.3	> 6.8	NT
C57BL/COM	ip	< 0.8	> 4.8	> 4.2	NT
C57BL/COM	ip	< 0.8	5.9	5.2	5.9
Lac/G/COM	iv	< 0.7	4.7	5.2	5.2

NT = Not tested.

* LD50 values are expressed as cfu/mouse.

Fig. 1. Plasmid profiles of *S. dublin* strains obtained by electrophoresis of extracts in agarose gel; mutant M173 is in lane 1 and cured derivative 173/2 in lane 2. The 46×10^6 -mol. wt plasmid and contaminating chromosomal DNA (chr.) are indicated.

Fig. 2. The accumulation of *S. dublin* strains 2229 (●—●) and M51 (●---●) in mouse spleens after oral inoculation. (A). Dose of strain 2229 was 6×10^5 cfu/mouse; mice did not survive after day 4. Dose of strain M51 was 5×10^5 cfu/mouse; all mice survived. (B). Dose of strain M51 was 3×10^7 cfu/mouse (LD₅₀ dose); 50% of mice survived. The arrow indicates the period during which mice died. After day 10 all surviving mice remained healthy.

their spleens after that time. Similar results were obtained with strain M173 (results not shown). The accumulation of both mutants in the spleens of mice showed that each could still invade across the gut mucosa. However, these results did not show whether invasion by the mutant strains was as efficient as that of the wild type.

Haemagglutinin production

Adhesion to, and invasion of, HeLa cells by *S. typhimurium* depends upon a mannose-resistant haemagglutinin (MRHA) (Jones and Richardson, 1981). Therefore we assayed MRHA and also mannose-sensitive haemagglutinins (MSHA) in strain 2229, mutants M51 and M173, and the cured derivative 173/2. MSHA was detected in each strain but MRHA was not, indicating that MRHA is not involved in the virulence of *S. dublin* strain 2229 and

Table II. The survival of *S. dublin* strains in normal rabbit and bovine serum

Source of serum	Final concentration of serum (%)	Percentage survival after 90 min* of strain				
		2229	M51	M173	173/2	14R525†
Rabbit	0	329	322	324	NT	470
	5	722	306	333	NT	0.3
	10	271	238	300	NT	1×10^{-4}
	20	277	307	336	NT	2×10^{-4}
	40	417	384	360	NT	$< 1 \times 10^{-4}$
	80	370	311	260	NT	$< 1 \times 10^{-4}$
Bovine	0	182	167	242	200	320
	80	29	34	17	34	$< 1 \times 10^{-4}$

NT = Not tested.

† Strain 14R525 was a plasmid-free serum-sensitive laboratory strain of *E. coli*.* Percentage survival = $\frac{\text{Number of bacteria at 90 min}}{\text{Number of bacteria at 0 min}} \times 100$.

that MSHA must be chromosomally encoded. *S. dublin* strain 2229 does not adhere to HeLa cells or to bovine brush borders or caecal epithelial cells (E. J. Manning, unpublished results).

LD50 values after ip or iv injection

To assess further the invasiveness of the mutants in mice, their virulence after ip and iv injection was determined. If the invasiveness of mutants M51 and M173 had been altered by the transposon insertion, their virulence after ip or iv injection, which bypasses the gut invasion step of pathogenesis, should have been more like that of the parent strain. However, there was still a 10^4 – 10^5 difference between the LD50 values of the wild-type and mutant strains (table I). This suggests that the reduced virulence of the mutants for mice when administered by mouth was not due to reduced invasion of the gut.

Serum resistance

Measurements were made *in vitro* of the serum resistance of strain 2229, mutants M51 and M173, and the cured derivative 173/2. All strains were equally resistant to rabbit serum; they grew in a final serum concentration of 80%. Each strain was slightly sensitive to bovine serum (table 2).

Discussion

Our virulence studies on *S. dublin* wild-type and

mutant strains have shown that the *S. dublin* plasmid of mol. wt 46×10^6 is important for the pathogenicity of the organism. These results are in agreement with those of other workers (Jones *et al.*, 1982; Terakado *et al.*, 1983). However, the plasmid does not seem to affect the ability of the organism to cross the mouse gut wall, because loss of virulence in the mutants was unaffected by the route of inoculation. This contrasts with the role of the *S. typhimurium* plasmid in invasion of HeLa cells (Jones *et al.*, 1982). The virulence of the cured derivative 173/2 was similar to that of mutants M173 and M51, indicating that the residual virulence of this cured derivative (and probably also of M51 and M173) is chromosomally-encoded.

The ip and iv routes of inoculation are highly efficient methods of establishing infection with *S. dublin* strain 2229 in mice. It was not possible to calculate an LD50 value for strain 2229 but a dose of < 5 cfu was always lethal (table 1). If only one or two organisms need to cross the gut wall to establish infection (Collins, 1972), it is perhaps not surprising that we have been unable to correlate virulence with adhesion to, or invasion of, intestinal cells and membranes. Indeed, invasion of the gut may be a passive process and an "invasive" organism may simply be one that can survive host defence processes once it has penetrated the gut wall.

The finding that each *S. dublin* strain tested showed mannose-sensitive, but not mannose-resistant, haemagglutinating activity indicated that MSHA is not plasmid encoded, and that MRHA is not involved in the virulence of *S. dublin* strain 2229 in mice. Tavendale *et al.* (1983) suggested that MSHA, and not MRHA, is necessary for the adhesion of *S. typhimurium* to HEp2 and HeLa cells *in vitro*. However, MSHA is not thought to be essential for infection (Duguid *et al.*, 1976; Tavendale *et al.*, 1983), therefore adhesion to cells *in vitro* may not be a good model for virulence *in vivo*. Although MRHA was not detected in strain 2229, c. 60% of all other *S. dublin* strains tested had MRHA activity against at least one species of erythrocyte (G. T. H. Brown and E. J. Manning, unpublished results). Thus strain 2229 differs in this respect from many other *S. dublin* isolates, and also from the *S. typhimurium* strains described by Jones and Richardson (1981).

Because our results suggested that the mutants had not lost their ability to cross the gut wall, it seemed possible that their reduced virulence might be due to diminished ability to resist host defences. One aspect of resistance to host defences is the ability of certain bacteria to resist the bactericidal action of normal serum (Taylor and Hughes, 1978)

but the wild-type and mutant strains were equally resistant to rabbit and to bovine serum. The serum resistance of *S. dublin* strain 2229 must be chromosomally encoded, unlike that of some *E. coli* strains (Taylor and Hughes, 1978), because results obtained with strains 2229 and 173/2 were similar. It is not known how well this in-vitro test relates to host defence mechanisms *in vivo*.

In conclusion this work has shown that in two

instances an insertion-mutation in the 46×10^6 -mol. wt plasmid of *S. dublin* reduces virulence of the organism, but apparently not by affecting its ability to cross the gut wall. The possibility that the mutants can less easily resist host defences *in vivo* requires further investigation.

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