In-vitro and in-vivo characteristics of *TnphoA* mutant strains of *Salmonella* serotype Gallinarum not invasive for tissue culture cells

P. A. BARROW, M. A. LOVELL and D. C. OLD*

AFRC Institute for Animal Health, Houghton Laboratory, Houghton, Huntingdon, Cambridgeshire PE17 2DA and

*Department of Medical Microbiology, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY

Summary. Insertion mutants of a strain of *Salmonella* serotype Gallinarum, the cause of fowl typhoid, were produced with transposon *TnphoA*. Eight mutants were identified as being less invasive in cell culture than the parent strain. Neither the parent strain nor the mutants showed mannose-sensitive haemagglutination of various red blood cell species. Although two mutants gave mannose-resistant (MR) haemagglutination of red cells of different animal species these MR activities could not be correlated with other characteristics *in vitro* or *in vivo*. The mutant strains were divisible into three classes by their patterns of invasiveness and adhesiveness *in vitro* and by changes in membrane proteins. Strains of classes 1 and 2 had single transposon insertions, detected by Southern hybridisation, of 8.9 and 2.4 kb *EcoRV*-digested chromosomal-fragments, respectively, and were slightly less invasive than the parent strain. They were no less virulent for chickens by the oral route than a mutant strain shown to be as invasive *in vitro* as the parent strain. The class-2 mutant was also less adhesive than the other strains. The single mutant strain of class 3 which contained insertions in several chromosomal fragments, was non-invasive in Vero cells ($< \log_{10}$ 1.0 cfu/ml recovered compared with $\log_{10}$ 2.88 for the parent strain) and showed reduced virulence by both oral and intramuscular routes. The mutant strains of all classes were taken up equally rapidly from the blood by the spleen. Intramuscular immunisation of chickens with the class-3 mutant strain gave complete protection against oral challenge 3 weeks later with 1000 oral LD50 doses of the virulent parent strain.

Introduction

Salmonella of serotype Gallinarum, the causative agent of fowl typhoid, is an avian pathogen of worldwide significance. The very high virulence of strains of Gallinarum for chickens is related to the possession of an 85-kb plasmid¹ involved in invasiveness *in vivo* but not *in vitro*, and to the ability of the strain to survive and multiply in cells of the reticulo-endothelial system. Chromosomal genes contribute to the full expression of these virulence characteristics in strains of Gallinarum² and are also responsible for its invasiveness in cultured cells.³

Invasiveness *in vivo* is an important virulence characteristic. For *Salmonella* serotypes such as Typhi, Dublin, Choleraesuis, Gallinarum and Abortusequi, which typically produce systemic disease, its importance is self evident in view of the usual faecal-oral route of transmission. For some other diseases, such as Typhimurium infection in young chickens, it is the virulence determinant of overriding importance.⁴ Work by Barrow *et al.*⁵ (and unpublished results)

indicates that Typhimurium is able to invade throughout the length of the alimentary tract.

Although invasion *in vitro* is not necessarily an accurate indication of invasiveness *in vivo*, an increased understanding of the microbial and molecular basis of invasion by another enteric pathogenic genus, *Shigella*, has resulted from studying invasion *in vitro* by the use of cultured monolayers.^{6–11} Unlike shigellae, little is known about the microbial determinants and cell receptors involved in salmonella invasion *in vitro*. Recent work has employed transposon mutagenesis of *Choleraesuis* with *TnphoA* (Tn5 1S50::phoA), a Tn5-derived transposon which allows detection of genes encoding exported proteins. This work has shown that several proteins are involved in invasion and translocation of bacteria across the cytoplasm of polarised epithelial cells.^{12,13} Using an in-vitro invasion system, Gálan and Curtiss¹⁴ have cloned a chromosomal invasion locus from Typhimurium; *TnphoA* was then used to identify three genes for invasiveness (*inv*) within that locus.

Greater understanding of the molecular basis of invasiveness in *Salmonella* serotypes may assist in explaining the varying degrees of virulence observed

with different strains of the same serotype.⁴ Such work may also contribute to our ability to create new vaccines for serotypes such as Gallinarum by producing non-invasive strains which, whilst useful for parenteral immunisation, would not be virulent by the natural route of infection.

Materials and methods

Bacterial strains

Gallinarum strain 9^{1,15} is highly virulent for chickens and produces typical fowl typhoid. Typhimurium strain F98 and *Escherichia coli* strain K12 prototroph were used as invasive and non-invasive control strains, respectively, in the cultured tissue-cell assay.³ Other strains of bacteria used only in constructing *TnphoA* mutant strains are described in the appropriate methods section below. Unless otherwise indicated, cultures were grown in 10 ml of Nutrient Broth (Oxoid CM67) and incubated at 37°C for 24 h in a shaking water-bath (100 strokes/min); such cultures contained (5×10^8) – (1×10^9) cfu/ml.

Construction of *TnphoA* mutant strains

TnphoA (Tn5 1S50_L::*phoA*) is a transposon probe designed to identify mutations in genes which code for proteins expressed at the cell surface.^{16,17} *TnphoA* contains the structural gene but not the signal sequence or promoter for alkaline phosphatase, an enzyme which is only active if secreted. When the transposon inserts into genes encoding secreted proteins, fusion polypeptides are produced which are hybrids of the polypeptide normally produced by the disrupted gene and of the alkaline phosphatase. Thus, selection for kanamycin resistance (Tn5) and alkaline phosphatase activity, indicated by dark blue colonies on an appropriate substrate, allows selection for insertion mutants in genes encoding secreted proteins.

Plasmid pRT733 (ori R6K, tra⁻, mob⁺, Ap⁺, Km⁺) containing *TnphoA* was transferred from *E. coli* strain SM10¹⁸ into a nalidixic acid-resistant mutant strain of Gallinarum strain 9 by conjugation on a plate surface at 30°C¹⁹ as described by Finlay *et al.*¹² Transcipients were selected on L agar containing sodium nalidixate 25 mg/L, kanamycin sulphate 45 mg/L and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidene salt (BCIP) 40 mg/L (Sigma). Dark blue colonies were checked for ampicillin sensitivity indicating loss of pRT733.

DNA methods

Southern-blot analysis of mutant and parent strains was performed by the following method. Chromosomal DNA was prepared by a modification of the method described by Maskell *et al.*²⁰ The pellet

harvested by centrifugation from 100 ml of a log-phase broth culture was resuspended in 2 ml of sucrose 25% w/v in ice-cold 50 mM Tris containing 1 mM EDTA, pH 8.0. Cells were lysed slowly for 5 min with lysozyme 1 g/L on ice, followed by proteinase K 500 mg/L, in 0.1 M EDTA (pH 8.0) and N-lauroyl-sarcosine (Sarkosyl) 2% w/v; the mixture was left on ice until lysis was complete and then incubated at 50°C overnight. After phenol extraction and ethanol precipitation, DNA was resuspended in 10 mM Tris, with 1 mM EDTA, pH 8.0. Restriction analysis, gel electrophoresis, nick translation and Southern blot analysis were performed as described by Sambrook *et al.*²¹ The plasmid content of strains was examined by the method of Kado and Liu.²²

Preparation of membrane proteins

Total-membrane protein preparations were made from 200-ml broth cultures shaken overnight. These were centrifuged at 7000 *g* for 10 min at 4°C. The pellet was washed twice in phosphate-buffered saline (PBS) and centrifuged again before resuspension in 10 ml of PBS containing 140 mM 2-mercaptoethanol. The suspension was sonicated for five 30-s periods with intervals to allow cooling. The sonicate was centrifuged at 15 000 *g* for 10 min at 4°C and the pellet was discarded. The supernate was centrifuged at 100 000 *g* for 60 min at 4°C; the pellet, resuspended in 1 ml of PBS, was centrifuged again at 100 000 *g* for 30 min at 4°C, and the pellet was resuspended in 1 ml of PBS. Polyacrylamide-gel electrophoresis (PAGE) of preparations was carried out in a standard way.²¹ *PhoA*-fusion products of mutant and parent strains were detected by non-reducing PAGE, visualising phosphatase activity with BCIP, as described by Finlay *et al.*¹²

Cell culture assays for invasiveness and adhesion

The invasiveness of bacterial strains for primary chicken kidney (CK) and Vero-cell monolayers was determined by the standard assay procedure described by Barrow and Lovell.³ The attachment of strains to cells was quantified with a modification of that procedure as follows. Broth cultures adjusted to E_{600} 0.7, were diluted 1 in 200 in Hanks's Balanced Salts Solution (HBSS). The cell lines were washed twice with HBSS before a 5-min period of contact with bacterial suspension. Monolayers were then washed five times before they were lysed and viable bacteria (cfu/ml) in the lysate counted. Each experiment was performed twice.

Haemagglutination

Salmonella strains were cultured in a number of ways: (i) on phosphate-buffered (pH 7.0) nutrient agar for 24 h at 37°C, harvesting bacterial cells in PBS to 10^{12} cfu/ml; (ii) in nutrient broth statically at 37°C

with subculture from the air-broth interface at intervals of 4–5 days, centrifuging each culture and resuspending the pellet in PBS to 5×10^{10} cfu/ml; (iii) in phosphate-buffered (pH 7.0) nutrient broth at 37°C for 4 days and suspended as in (ii). For most cultures, haemagglutination tests were performed as described by Old²³ on porcelain tiles with washed red blood cells from fowl, guinea-pig, horse, man, pig and sheep, with and without the presence of α -D-methylmannoside 0.1% w/v. For the second type of broth culture in (iii), settling tests at 4°C in microtitration trays were also performed.

Experimental animals

Unless otherwise indicated, chickens were unsexed Light Sussex from a specified pathogen-free (SPF) flock held at this Institute. The methods and the diet used have been described.²⁴ All feed was unmedicated.

Virulence and pathogenicity studies

Two-week-old chickens were used for oral and intramuscular virulence estimations. Chickens were inoculated orally with undiluted broth cultures (0.3 ml), or with 0.1-ml volumes of serial dilutions of broth cultures in nutrient broth via the gastrocnemius muscle. The number of deaths over a 3-week period was recorded and the percentage mortality and LD50 were estimated, the latter by the Maximum Likelihood Programme statistical package (AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden) which follows conventional methods of probit analysis.²⁵

To assess uptake by the reticulo-endothelial system, groups of 24 2-week-old chickens were inoculated intravenously with 0.1 ml of a 1 in 10^4 dilution of broth culture of mutant or parent strains. At intervals, three chickens from each group were killed and viable counts were estimated for samples of cardiac blood and spleen by the methods described above.

Invasiveness in vivo

For an estimation of bacterial invasion, groups of 12 2-week-old chickens were inoculated orally with 0.3 ml of a broth culture of each strain. At intervals after inoculation, three birds from each group were killed and viable counts were determined for liver, spleen, caecal contents and caecal epithelium; the latter sample was prepared as described by Barrow *et al.*⁵ Viable counts were made by the method of Miles *et al.*,²⁶ with dilutions plated on to Brilliant Green Agar (Oxoid CM263) containing sodium nalidixate 20 mg/L and novobiocin 1 mg/L.

Assessment of immunogenicity

Fifty 12-week-old SPF chickens (Rhode Island Red) were inoculated intramuscularly with 10^8 bacteria

(0.1 ml) of one of the *TnphoA* mutant strains. Three weeks later, these chickens and an uninoculated group of chickens of the same age were inoculated orally with 10^7 bacteria (in 0.3 ml) of Gallinarum strain 9 NaI^r. Mortality was monitored daily.

Results

In-vitro adhesiveness and invasiveness of mutant strains with chicken kidney and Vero cells

Two hundred colonies, identified by ampicillin sensitivity and by their expression of alkaline phosphatase as *TnphoA* insertion mutants, were tested for their invasiveness for CK and Vero cells. The *in-vitro* invasiveness results are shown together with those of a *S. typhimurium* invasive control and an *E. coli* non-invasive control (table I) and are summarised in table II for the parent strain, a mutant strain (238) as invasive as the parent strain, eight mutant strains less invasive than the parent strain and for the control strains. Three classes of mutant strains were identified: *class 1* consisted of six mutant strains, all of which were of reduced invasiveness for both cell lines; *class 2* comprised only strain 89 which was of reduced invasiveness for CK cells but almost as invasive as the parent strain in Vero cells; *class 3* also comprised only one strain (196), which showed reduced invasiveness for CK cells and greatly reduced invasiveness for Vero cells ($< \log_{10} 1.0$ cfu/ml) (table I).

From a short contact-time experiment, which allowed attachment to occur without invasion, it was apparent by comparison with the parent strain that all class-1 mutant strains were slightly less adhesive for both Vero and CK cells, whereas the class-2 mutant strain 89 was markedly less adhesive in CK cells ($\log_{10} 1.95$ compared with $\log_{10} 2.56$). The class-3 mutant strain 196 was as adhesive as the parent strain (table I). Very similar results were obtained when the experiment was repeated. The method has been shown to be reproducible under the conditions used.³

Haemagglutination

After growth in phosphate-buffered broth or phosphate-buffered agar, the parent Gallinarum strain 9 and the mutant strains derived from it gave neither mannose-sensitive nor mannose-resistant haemagglutination, i.e., they were MSHA⁻ MRHA⁻. When grown serially in unbuffered nutrient broth, these Gallinarum strains were again MSHA⁻ but two strains gave MRHA reactions. The parent strain 9 gave MRHA of horse red blood cells, a weak reactivity detectable in microtitration plates but not in tile tests. The mutant strain 186 (of invasion-class 1) produced in each of the three nutrient broth cultures an MRHA active against sheep red blood cells and detectable by the tile test. When these same (nutrient broth)

Table I. Invasiveness and adhesiveness *in vitro* of *Salmonella* Gallinarum parent strain 9 and *TnphoA*-insertion mutant strains for chicken kidney and Vero cells

Strain no.	Viable count (log ₁₀ cfu/ml) of cell lysate when bacteria were tested in cells of				In-vitro invasion class
	chicken kidney for		Vero for		
	invasion	adhesion	invasion	adhesion	
Gallinarum					
9	3.41	2.56	2.88	2.85	WT
238	3.49	2.70	2.83	2.90	inv ⁺
32	1.65	2.38	1.98	2.38	I
66	1.60	2.32	1.78	2.56	
111	1.60	2.44	1.88	2.67	
186	1.54	2.34	1.88	2.42	
194	1.39	2.42	1.60	2.30	
230	1.93	2.34	1.82	2.46	
89	1.39	1.95	2.30	2.26	2
196	1.78	2.65	<1.0	2.71	3
Typhimurium* F98	4.86	4.02	5.45	3.85	inv ⁺
<i>E. coli</i> * K12	<1.0	3.16	<1.0	2.72	inv ⁻

WT, wild type strain.

* Control strains that are invasive (F98) and non-invasive (K12).

suspensions were tested by the more sensitive microtitration method, the spectrum of MRHA activity of strain 186 broadened and, whilst reactions were still strongest with sheep red cells, those of horse, guinea-pig and man were also agglutinated.

Southern blot analysis

Chromosomal DNA was prepared from Gallinarum strain 9, from the 8 *TnphoA* mutant strains identified as less invasive *in vitro* for CK or Vero cells and from the one *TnphoA* invasive mutant strain 238. DNA was digested with *EcoRV* (which does not cut within the *TnphoA* transposon), electrophoresed, transferred to nitrocellulose and probed with radiolabelled pRT733 DNA, the donor plasmid containing *TnphoA*. DNA from the parent strain did not hybridise with the probe (fig. 1). Each of the six mutant strains comprising invasion-class 1 contained a single chromosomal fragment of 16.5 kb which hybridised with the probe, whereas the class-2 strain contained a single fragment of 10 kb (lane 4). The class-3 mutant strain (lane 8) contained five fragments homologous to the probe, presumably as a result of duplication following co-integrate-formation between chromosome and donor plasmid. *TnphoA* has a size of 7.6 kb suggesting that, in the case of class-1 and -2 mutants, insertion had occurred in chromosomal fragments of 8.9 kb and 2.4 kb size, respectively. The invasive mutant strain 238 had two inserts into fragments of sizes 16.0 kb and 8.6 kb. The results are summarised in table II.

All the mutant strains possessed the 85-kb virulence-associated plasmid,¹ known to contribute to invasive-

ness in the chicken but not necessary for full expression of invasion of Vero cells *in vitro*.³

All the mutant strains possessed smooth lipopolysaccharide, as shown by agglutination with O9-specific antiserum and by the absence of agglutination by acriflavine.

PAGE analysis of mutant strains and characterisation of *PhoA*-fusion products

Sodium dodecyl sulphate-PAGE was performed on total membrane preparations from the parent strain and the non-invasive mutant strains and proteins were visualised with a silver stain (fig. 2). All the mutant strains possessed bands, of sizes 16, 35 and 56 Kda, not possessed by the parent. The latter two, at least, are known to be specified by *TnphoA*. Mutant strains 111, 186, 194 and 230 (designated class 1b) possessed an additional band of 48 Kda and mutant strain 89 (class 2) a band of 63 Kda. The 16- and 35-Kda bands were also detectable by staining with Coomassie Brilliant Blue. A very faint band of 16 Kda was also seen in the parent strain. It was not possible to determine whether this was the same or a different protein.

Mol.-wt estimations were extremely difficult with the non-reducing gel on which alkaline phosphatase activity was detected in order to visualise *PhoA*-fusion proteins (fig. 3). Again, mutant strains 111, 186, 194 and 230 (class 1b, lanes 4, 5, 6 and 8) showed identical patterns, with two bands with an estimated size of approximately 72 Kda. Different patterns involving single bands of c. 71 Kda—mutant 89 (class 2) and

Table II. *Salmonella* Gallinarum strain 9 and Tnp*hoA*-insertion mutant strains

Strain no.	Insert in DNA fragment (kb)	Adhesion <i>in vitro</i> (table I)	Invasion <i>in vitro</i> in cells of		Size (Kda) of mutant-specific protein bands in gel that was		Virulence for chickens (table III)	Invasion <i>in vivo</i> (table IV)	Uptake by spleen*	In-vitro invasion class
			CK	Vero	non-reducing	reducing				
9	...	+	+	+	...	...	+	+	+	WT
238	16.0, 8.6	+	+	+	...	...	±	+	+	inv +
32	8.9	+	-	±	70	16, 35, 56	...	...	...	1a
66	8.9	+	-	±	65, 68	16, 35, 56	±	+	+	
111	8.9	+	-	±	72, 73	16, 35, 48, 56	...	...	...	1b
186	8.9	+	-	±	72, 73	16, 35, 48, 56	...	...	...	
194	8.9	+	-	±	72, 73	16, 35, 48, 56	+	+	+	
230	8.9	+	-	±	72, 73	16, 35, 48, 56	...	...	...	
89	2.4	±	-	+	71	16, 35, 56, 63	±	±	+	2
196	8-14.5	+	-	-	71	16, 35, 56	-	-	+	3

WT, wild type strain.

* After intravenous inoculation.

+, Positive reaction or presence of character; ± slight reduction in reaction or character; -, strong reduction or elimination of character; ... not done;

Fig. 1. Autoradiograph of a Southern blot from a gel of separated chromosomal fragments from Gallinarum strain 9 and *TnphoA* insertion mutant strains digested with *EcoRV* and probed with ^{32}P -labelled pRT733. Lane 1, parent strain 9; 2-10, mutant strains 32, 66, 111, 186, 194, 196, 230, 238. The position of mol.-wt markers in kb is shown (MW).

196 (class 3), lines 7 and 9—or smaller bands were seen with the other mutant strains. The results are presented in summary form in table II.

Virulence for chickens

The virulence for chickens of Gallinarum strain 9 and the *TnphoA* mutant strains, when inoculated

Fig. 2. SDS-PAGE of membrane protein preparations from Gallinarum strain 9 and *TnphoA* insertion mutant strains. Lanes 1-8, mutant strains 32, 66, 89, 111, 186, 194, 196, 230; 9, parent strain. Lane MW shows mol.-wt markers with the size (Kda) indicated; • indicates proteins not found in the parent strain.

Fig. 3. Non-reducing PAGE of bacterial sonicates stained with nitroblue tetrazolium and BCIP to indicate alkaline phosphatase activity. Lanes 1-9, mutant strains 32, 66, 89, 111, 186, 194, 196, 230, 238; 10, parent strain. Lane AP is alkaline phosphatase. Mol.-wt markers (Kda) are indicated.

orally or intramuscularly, is shown in tables II and III. The invasive mutant strain 238 was slightly less virulent than the parent strain by both routes of inoculation. The two class-1 mutant strains (66 and 194) tested were not significantly less virulent than the parent strain by the oral route and had similar LD50 values. The virulence of the class-2 mutant strain 89 was similar to that of the parent strain. The class-3 mutant strain 196 was almost avirulent; it produced few deaths (one out of 34) after oral inoculation and had a very high LD50 value after intramuscular inoculation (table III).

Invasiveness in vivo

The ability of the parent strain and selected mutant strains to invade the caecal wall and localise in the liver and spleen of chickens is shown in table IV and summarised in table II. For each strain, similar numbers of bacteria were isolated from the caecal contents. The numbers of bacteria of the parent strain isolated from the caecal epithelium increased with time and the numbers in the caecal contents declined to a point at which more bacteria were isolated from the wall than from the contents, i.e., it indicated invasiveness. Mutant strains 238 (invasive) and 66 (designated 1a) showed a similar, albeit slightly delayed, pattern of invasiveness. The other mutant strains did not show an increase in the number of organisms isolated from the caecal epithelium. Individual birds showed detectable numbers in the tissues that were higher in the case of mutant strain 194 (class 1b) than of strains 89 or 196 (classes 2 and 3). Bacteria of the parent strain, the invasive mutant strain 238 and mutant strain 66 were isolated in similar numbers from the liver and spleen by 2 days after infection. Recovery of mutant strain 194 (class 1b) from these organs was somewhat delayed. Mutant strain 89 (class 2) was recovered from liver and spleen in individual birds whereas mutant strain 196 (class 3) was not detected at all (table IV).

Table III. Virulence for chickens following oral or intra-muscular inoculation of *Salmonella* Gallinarum strain 9 and *TnphoA*-insertion mutant strains

Gallinarum strain no.	Invasion class*	Virulence of strain when chickens were inoculated			
		by oral route†			by intramuscular route†
		Number dead/ number tested (%)	χ^2	p value	Log ₁₀ LD50 (SEM)
9	WT	33/34 (97)			1.69 (0.27)
238	inv ⁺	21/34 (62)	6.48	0.015	2.70 (1.32)
66	1a	23/34 (68)	5.05	0.03	1.13 (0.29)
194	1b	29/34 (85)	2.92	0.20	1.66 (0.76)
89	2	25/34 (74)	1.74	0.08	1.77 (0.56)
196	3	1/34 (3)	60.23	<0.01	7.36 (0.41)

χ^2 and *p* values by comparison with value for parent strain 9. WT, wild type strain.

* See tables I and II.

† Two-week-old chickens were inoculated orally with 10⁸ bacteria (in 0.3 ml); groups of five 2-week-old chickens were inoculated via gastrocnemius muscle with decimal dilutions of broth cultures (in 0.1 ml).

Table IV. In-vivo invasiveness and association with the caecal wall of Gallinarum parent strain 9 and *TnphoA*-insertion mutant strains in chickens inoculated orally

Time (h) after inoculation*	Strain	Invasion class†	Viable count (log ₁₀ cfu) of salmonellae/g of tissue in			
			liver	spleen	caecum	
					contents	wall
12	9	WT	N	N	4.5	2.5
	238	inv ⁺	N	N	3.9	N(3.2)
	66	1a	N	N	4.7	N(2.3)
	194	1b	N	N	4.1	2.7
	89	2	N	N	3.5	N(2.9)
	196	3	N	N	3.6	N(2.0)
24	9	WT	N	N	3.5	3.6
	238	inv ⁺	N	N	N	N(2.0)
	66	1a	N	N	2.7	N(2.0)
	194	1b	N	N	2.8	N(3.3)
	89	2	N	N	3.6	N
	196	3	N	N	3.6	N(2.0)
48	9	WT	2.0	2.6	3.4	3.3
	238	inv ⁺	3.9	4.4	N	3.3
	66	1a	2.0	2.9	3.3	2.8
	194	1b	N	N(2.0)	N(2.7)	N(4.2)
	89	2	N(3.1)	N(4.2)	N	N
	196	3	N	N	N(2.3)	N(2.0)
72	9	WT	4.7	4.6	2.7	4.3
	238	inv ⁺	4.8	5.0	2.3	4.5
	66	1a	3.9	4.5	2.5	2.3
	194	1b	2.5	3.3	N(2.5)	N(3.5)
	89	2	N(3.5)	N(4.2)	N(2.0)	N
	196	3	N	N	N	N(2.3)

N, <2.0.

WT, wild type parental gallinarum strain.

* Chickens were inoculated orally with 10⁸ bacteria (in 0.3 ml).

† See table II; median count, with three chickens tested (highest value in parentheses).

Uptake by the spleen

The parent strain and all mutant strains were taken up rapidly from the blood into the spleen after intravenous inoculation so that bacteria were not detectable in the blood 30 min after inoculation (data not shown in detail but summarised in table II).

Effect of immunisation on survival of chickens after challenge

After challenge of unimmunised control birds with the fully virulent parent strain, 15 of 25 birds died between 7 and 15 days after challenge, all exhibiting signs of acute fowl typhoid. Chickens which died

showed enlarged bronze coloured livers and had enlarged spleens. On the other hand, none of the birds immunised parenterally with TnphoA mutant strain 196 died when similarly challenged with the same parent strain of Gallinarum. When these birds were examined *post mortem* at the end of the experiment, liver and spleen appeared completely normal.

Discussion

TnphoA has proved to be a useful molecular tool in studying the basis of virulence in organisms such as *Salmonella* of serotypes Choleraesuis^{4, 12} and Typhimurium,¹⁴ *Vibrio cholerae*¹⁷ and *Rhizobium meliloti*²⁷. Few problems were encountered in its use with serotype Gallinarum although care had to be exercised to ensure the selection of dark blue colonies because some variation in colour intensity is always observed.²⁸ More problems have been encountered with serotype Typhimurium (unpublished results) because of a more intense background, greater colour variation in colonies together with instability of the Km⁺PhoA⁺ phenotype.

Several mutant strains classified as group 1b were identified, suggesting that TnphoA, like Tn5 from which it was constructed, has preferential sites of insertion, i.e., "hot spots".²⁹ A similar observation was made with Choleraesuis.¹² In the case of only one mutant strain (196) was invasiveness *in vitro* in Vero cells reduced to the level of the "non-invasive" *E. coli* strain K12 prototroph. For the other mutant strains, invasiveness was reduced by between 10- and 100-fold, levels consistent with previous findings for Choleraesuis.¹²

The mutant strains behaved differently in the two cell lines. In a previous study³ the invasiveness of *Salmonella* strains was virtually identical in different cell lines. The present results were surprising and suggested that the receptors for adhesiveness and invasion or both might be different in different cell types. The production of a fusion protein between the phoA gene product and the adhesion or invasion gene product may have altered the configuration of the latter thereby preventing association with the receptor on the chicken kidney cell surface but not changing the association with a less discriminating or different receptor on the Vero cell surface.

With the exception of mutant strain 89, all mutant strains were phenotypically fully adhesive. Mutant strain 89 appears to be equivalent to the less invasive, less adhesive class 1e mutant characterised by Finlay *et al.*¹² whose results, however, contrast with ours in that they did not find an adhesive, non-invasive type of mutant. An examination of more PhoA⁺ clones of

Gallinarum might have generated more mutant strains of the less adhesive kind. Mutant strain 89 also differed from the other mutant strains in its membrane proteins because it possessed an additional mutant-specific protein of 63 Kda. Although there was no difference in the haemagglutinating properties of strain 89, it is conceivable that this protein is associated in some way with adhesiveness. The true size of such proteins is difficult to estimate because, in the formation of a fusion protein, the entire amino-acid sequence of the disrupted gene may not be expressed. Although this mutant strain was also less adhesive or invasive, or both, than the other strains (except 196) *in vivo*, all of them were virulent to some degree for chickens. The level of adhesiveness, although reduced, may have been sufficient to allow invasion to occur. Another possibility is that adhesiveness and invasiveness are properties that are not linked.

The other new proteins observed to be associated with reduced invasiveness (other than the TnphoA-associated 35- and 56-Kda proteins) were a 16-Kda protein present in all mutant strains and a 48-Kda protein present in class-1b mutant strains. This latter protein may be a truncated version of the 54-Kda *invA* protein described by Galán and Curtiss.¹⁴ A protein of 16 Kda has not been described before.

Despite the differences in invasiveness *in vivo*, all mutant strains were taken up by the spleen with equal rapidity when chickens were inoculated intravenously. This could not have been due to the presence of opsonic antibodies because the chickens were *Salmonella*-free, a finding which suggests that intestinal invasiveness and uptake by the reticulo-endothelial system, probably by macrophages, may involve different mechanisms.

The virulence and invasiveness *in vivo* of mutant strains identified *in vitro* as "less invasive" emphasises the need for in-vivo screening in such studies. If in-vitro invasion does reflect in-vivo epithelial cell penetration, an explanation of the results could be that mucosal penetration *via* this route may not be the only mechanism for invasion. Thus, mutants unable to penetrate the mucosa in this way might nevertheless enter the sub-mucosa, e.g., *via* M cells. This interpretation would be supported by the equally rapid uptake by the spleen of all the mutants tested. Although mutant strain 196 was much less virulent and invasive, it was highly protective when chickens were inoculated parenterally, indicating that strains which are avirulent when inoculated parenterally and are of reduced invasive ability may be potential candidates as live, attenuated vaccines.

We thank Mr B. Wells for looking after the experimental animals and Miss V. Peters for secretarial assistance.

References

1. Barrow PA, Simpson JM, Lovell MA, Binns MM. Contribution of *Salmonella gallinarum* large plasmid toward virulence in fowl typhoid. *Infect Immun* 1987; **55**: 388-392.
2. Barrow PA, Lovell MA. Functional homology of virulence plasmids in *Salmonella gallinarum*, *S. pullorum*, and *S. typhimurium*. *Infect Immun* 1989; **57**: 3136-3141.
3. Barrow PA, Lovell MA. Invasion of Vero cells by *Salmonella* species. *J Med Microbiol* 1989; **28**: 59-67.
4. Barrow PA, Huggins MB, Lovell MA, Simpson JM. Observations on the pathogenesis of experimental *Salmonella*

- typhimurium* infection in chickens. *Res Vet Sci* 1987; **42**: 194-199.
5. Barrow PA, Simpson JM, Lovell MA. Intestinal colonisation in the chicken by food-poisoning *Salmonella* serotypes; microbial characteristics associated with faecal excretion. *Avian Path* 1988; **17**: 571-588.
 6. Sansonetti PJ, Kopecko DJ, Formal SB. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect Immun* 1982; **35**: 852-860.
 7. Sansonetti PJ, Hale TL, Dammin GJ, Kapfer C, Collins HH, Formal SB. Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. *Infect Immun* 1983; **39**: 1392-1402.
 8. Hale TL, Sansonetti PJ, Schad PA, Austin S, Formal SB. Characterization of virulence plasmids and plasmid-associated outer membrane proteins in *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*. *Infect Immun* 1983; **40**: 340-350.
 9. Watanabe H, Nakamura A. Identification of *Shigella sonnei* form 1 plasmid genes necessary for cell invasion and their conservation among *Shigella* species and enteroinvasive *Escherichia coli*. *Infect Immun* 1986; **53**: 352-358.
 10. Baudry B, Maurelli AT, Clerc P, Sadoff JC, Sansonetti PJ. Localization of plasmid loci necessary for the entry of *Shigella flexneri* into HeLa cells, and characterization of one locus encoding four immunogenic polypeptides. *J Gen Microbiol* 1987; **133**: 3403-3413.
 11. Clerc P, Sansonetti PJ. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. *Infect Immun* 1987; **55**: 2681-2688.
 12. Finlay BB, Starnbach MN, Francis CL *et al*. Identification and characterization of *TnphoA* mutants of *Salmonella* that are unable to pass through a polarized MDCK epithelial cell monolayer. *Mol Microbiol* 1988; **2**: 757-766.
 13. Finlay BB, Heffron F, Falkow S. Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. *Science* 1989; **243**: 940-943.
 14. Galán JE, Curtiss R. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. *Proc Natl Acad Sci USA* 1989; **86**: 6383-6387.
 15. Smith HW. Observations on experimental fowl typhoid. *J Comp Pathol Therapeutics* 1955; **65**: 37-54.
 16. Manoil C, Beckwith J. *TnphoA*: a transposon probe for protein export signals. *Proc Natl Acad Sci USA* 1985; **82**: 8129-8133.
 17. Taylor RK, Miller VL, Furlong DB, Mekalonos JJ. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci USA* 1987; **84**: 2833-2837.
 18. Simon R, Priefer U, Pühler A. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Biotechnology* 1983; **1**: 784-790.
 19. Bradley DE, Taylor DE, Cohen DR. Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. *J Bacteriol* 1980; **143**: 1466-1470.
 20. Maskell DJ, Morrissey P, Dougan G. Cloning and nucleotide sequence of the *aroA* gene of *Bordetella pertussis*. *J Bacteriol* 1988; **170**: 2467-2471.
 21. Sambrook J, Fritsch EF, Maniatis J. Molecular cloning—a laboratory manual, 2nd edn. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press. 1989.
 22. Kado CI, Liu S-T. Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* 1981; **145**: 1365-1373.
 23. Old DC. Haemagglutination methods in the study of *Escherichia coli*. In: Sussman M (ed) The virulence of *Escherichia coli*: reviews and methods. (Special publications of the Society for General Microbiology 13) London, Academic Press. 1985: 287-313.
 24. Smith HW, Tucker JF. The effect of antibiotic therapy on the faecal excretion of *Salmonella typhimurium* by experimentally infected chickens. *J Hyg* 1975; **75**: 275-292.
 25. Finney DJ. Assays based on quantal responses. In: Finney DJ, Statistical methods in biological assay. London, Charles Griffin and Co., Ltd. 1964: 468-490.
 26. Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. *J Hyg* 1938; **38**: 732-749.
 27. Long S, McCune S, Walker GC. Symbiotic loci of *Rhizobium meliloti* identified by random *TnphoA* mutagenesis. *J Bacteriol* 1988; **170**: 4257-4265.
 28. Miller I, Maskell D, Hormaeche C, Johnson K, Pickard D, Dougan G. Isolation of orally attenuated *Salmonella typhimurium* following *TnphoA* mutagenesis. *Infect Immun* 1989; **57**: 2758-2763.
 29. Berg DE, Berg CM. The prokaryotic transposable element Tn5. *Biotechnology* 1983; **1**: 417-435.