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The Association Between a Large Molecular Mass Plasmid and Virulence in a Strain of *Salmonella pullorum*

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Eight strains of *Salmonella pullorum* isolated from epidemiologically independent cases of pullorum disease (bacillary white diarrhoea) in young chickens possessed at least one large molecular mass plasmid in addition to smaller molecular mass plasmids. The 85 kb large plasmid, designated pBL001, of one of these strains was 'tagged' with an ampicillin resistance marker by the insertion of transposon Tn*3*. The plasmid was eliminated by passage in nutrient broth containing acridine orange. It was reintroduced into the strain from which it had been eliminated by mobilization using the F plasmid. Following oral inoculation of newly hatched Rhode Island Red chickens, the parent strain produced a high level of mortality (71%) with characteristic signs of pullorum disease. Following intramuscular inoculation of chickens of the same age, the bacterial LD_{50} was ($\log_{10}$ c.f.u.) $3{\cdot}38 \pm 0{\cdot}43$ (mean ± SEM). The derivative lacking pBL001 produced no mortality or morbidity when inoculated orally and the bacterial LD_{50} value increased to ($\log_{10}$ c.f.u.) $5{\cdot}54 \pm 0{\cdot}28$. This increase was statistically significant ($\chi^2 = 13{\cdot}6$, $P < 0{\cdot}01$). Reintroduction of pBL001 restored virulence as gauged by oral inoculation of chickens (62% mortality) and by the intramuscular bacterial LD_{50} value ($\log_{10}$ c.f.u. $= 3{\cdot}78 \pm 0{\cdot}25$). These values were not significantly different to those produced by the parent strain ($\chi^2 = 0{\cdot}59$, $P = 0{\cdot}4$ and $\chi^2 = 0{\cdot}66$, $P = 0{\cdot}5$, respectively). Following oral inoculation, the pBL001-cured derivative was less invasive than the parent strain and following intramuscular inoculation it persisted for a shorter period than the parent strain in the liver, spleen and the leg muscle into which it had been inoculated. In addition, the parent strain, but not the pBL001-cured derivative, localized in large numbers in the myocardium where it produced lesions typical of pullorum disease. Both the parent strain and the pBL001-cured derivative were serum resistant in the presence of rabbit serum and grew equally well in chick serum and broth.

INTRODUCTION

Recent studies have indicated that large molecular mass plasmids are required for the virulence of several serotypes of *Salmonella* which are capable of producing systemic disease in animals and man. These include *Salmonella typhimurium*, *S. dublin*, *S. enteritidis*, *S. cholerae-suis*, *S. paratyphi* C and *S. abortus-ovis* (Jones *et al.*, 1982; Terakado *et al.*, 1983; Popoff *et al.*, 1984; Baird *et al.*, 1985; Helmuth *et al.*, 1985; Nakamura *et al.*, 1985). The importance of an 85 kb plasmid in the virulence of *S. typhimurium* was demonstrated by Jones *et al.* (1982), who showed that curing the plasmid produced strains which were less virulent for mice; reintroduction of the plasmid restored virulence. A similar relationship between virulence for the mouse and possession of an 80 kb plasmid by *S. dublin* was demonstrated by Chikami *et al.* (1985) by curing and reintroduction of the plasmid. Studies with *S. enteritidis* relied only on the elimination of the large plasmid to demonstrate an association with virulence (Nakamura *et al.*, 1985).

Abbreviation: RIR, Rhode Island Red.

A recent study (Barrow *et al.*, 1987*b*) showed that an 85 kb plasmid present in *S. gallinarum* was responsible for the ability of this strain to produce fowl typhoid, an economically important disease of poultry. This work indicated that the plasmid was responsible for the ability of this serotype to invade via the alimentary tract and also to survive and grow in the cells of the reticuloendothelial system. In contrast, the invasiveness of *S. dublin* (Manning *et al.*, 1986) and of *S. typhimurium* (Hackett *et al.*, 1986; Pardon *et al.*, 1986; Gulig & Curtiss, 1987) is thought to be chromosomally mediated.

Salmonella pullorum and *S. gallinarum* are generally regarded as biotypes of the same serotype. *S. pullorum* produces 'pullorum disease', otherwise known as bacillary white diarrhoea, in young and, to a lesser extent, adult chickens, with considerable mortality and reduced egg production (Gordon & Jordan, 1982). Barrow *et al.* (1987*b*) found that one strain of *S. pullorum* had a large molecular mass plasmid which showed considerable homology with the 85 kb plasmid of *S. gallinarum*. The present study investigated an association between this plasmid and the ability of the *S. pullorum* strain to produce bacillary white diarrhoea in chickens. The role of the plasmid in the pathogenesis of the disease is also reported.

METHODS

Bacterial strains. Eight strains of *S. pullorum* were isolated from epidemiologically separate cases of bacillary white diarrhoea in chickens. They were maintained on Dorset egg slopes at 4 °C. One of them, *S. pullorum* strain 3, was virulent for chickens when inoculated by the oral route (Smith & Tucker, 1980). In experiments in which *S. pullorum* organisms were inoculated orally, spontaneous chromosomal mutants resistant to nalidixic acid (Nal^r), produced by the method of Smith & Tucker (1980), were used. These authors showed that Nal^r mutants of *Salmonella* strains were no less virulent for chickens than were the parent strains. The designation of *S. pullorum* strain 3 and its derivatives produced by procedures described below are shown in Table 1. Broth cultures were grown in 10 ml nutrient broth (Oxoid CM1) in a shaking water bath (100 strokes min^{-1}) at 37 °C for 24 h. These usually contained between 5×10^8 and 1×10^9 c.f.u. ml^{-1}.

Chickens. These were from a specified-pathogen-free Rhode Island Red (RIR) flock. Their sex was not determined. Their rearing conditions and diet were described by Smith & Tucker (1975).

Plasmid isolation. DNA was visualized by the method of Hansen & Olsen (1978). Electrophoresis of plasmid DNA was done using 0·7% agarose gels.

The size of the large plasmid was estimated by direct comparison with the 85 kb plasmid of *S. typhimurium*, the 70 kb plasmid of *S. dublin* and the 85 kb plasmid of *S. gallinarum* (Barrow *et al.*, 1987*b*). The size of small plasmids was estimated by comparison with the 2·5 kb plasmid in *S. gallinarum* 9 (Barrow *et al.*, 1987*b*).

Transposon mutagenesis and plasmid curing. To assist in curing, the large plasmid in strain 3 (designated pBL001) was 'tagged' with transposon Tn*3*, coding for ampicillin resistance. A temperature-sensitive tetracycline resistance (Tet^r) plasmid containing Tn*3* (pMB501; Binns *et al.*, 1985) was introduced into *S. pullorum* strain 3 by transformation (Kushner, 1978). After incubation at a restrictive temperature (42 °C) the insertion of the transposon into the large plasmid was verified by the ampicillin resistance (Amp^r) and tetracycline sensitivity

Table 1. *S. pullorum strain 3 and its derivatives*

Designation of strain	Properties/derivation	Possession of large molecular mass plasmid
3	Parental field strain (Smith & Tucker, 1980). Amp^s Nal^s	pBL001
3 Nal^r	A Nal^r mutant of strain 3 used in experiment shown in Table 3	pBL001
3(Tn*3*)	Strain 3 with pBL001 tagged by Tn*3*. Amp^r Nal^s	pBL001::Tn*3*
3(Tn*3*) Spc^r	A spontaneous chromosomal Spc^r mutant of strain 3(Tn*3*). Amp^r Nal^s	pBL001::Tn*3*
3(pBL001$^-$)	Strain 3(Tn*3*) from which pBL001 had been cured by acridine orange. Amp^r Nal^s	Not present (pBL001$^-$)
3(pBL001$^-$) Nal^r	A Nal^r mutant of strain 3(pBL001$^-$)	Not present (pBL001$^-$)
3(Tn*3*) Nal^r	Strain 3(pBL001$^-$) Nal^r into which pBL001::Tn*3* had been introduced from strain 3(Tn*3*) Spc^r by mobilization with the F plasmid. Amp^r Nal^r	pBL001::Tn*3*

(Tet^s) of the host, coupled with no apparent increase in the molecular mass of the small plasmid. The strain thus produced was designated 3(Tn*3*).

The Tn*3*-labelled plasmid (pBL001::Tn*3*) in this strain was cured by overnight growth in nutrient broth containing 600 μg acridine orange ml^{-1}. Plasmid loss was verified by sensitivity to ampicillin and by electrophoresis. This strain was designated 3(pBL001$^-$).

Bacterial mating procedures. To reintroduce pBL001 into strain 3(pBL001$^-$), a three-factor mating was used. The F plasmid present in a prototrophic *E. coli* K12 strain (proto) (Smith, 1978) was used to mobilize pBL001::Tn*3* from strain 3(Tn*3*) Spc^r (spectinomycin resistant) into a Nal^r (nalidixic acid resistant) mutant of *S. pullorum* strain 3(pBL001$^-$). Mating was done by incubation at 37 °C for 18 h followed by a further 8 h at 20 °C; the mating mixture was then plated on L agar (Lennox, 1955) containing ampicillin (100 μg ml^{-1}) and sodium nalidixate (20 μg ml^{-1}). Colonies were checked for spectinomycin sensitivity to ensure that they were not spontaneous Nal^r mutants of the intermediate donor. They were also tested for their sensitivity to the F-specific bacteriophage MS2 (Davis *et al.*, 1961) and for agglutination by acriflavine (Smith, 1965*b*) and O-specific antisera (Burroughs-Wellcome, UK).

Virulence and pathogenicity studies. Newly hatched RIR chickens (less than 24 h old) were used for oral and intramuscular virulence assessments. Oral inoculation of undiluted broth cultures and intramuscular inoculation of decimal dilutions of broth cultures were done as described previously (Barrow *et al.*, 1987*b*). The numbers of deaths over a four week period were recorded and the percentage mortality and LD_{50} were calculated. The LD_{50} values were calculated using the MLP statistical package (Rothamsted Experimental Station, Harpenden, UK), which follows conventional methods of probit analysis (Finney, 1964). Oral LD_{50} values were not calculated since the alimentary flora of the newly-hatched chicken is simple (Smith, 1965*a*), allowing small numbers of micro-organisms to multiply extensively, thus making an LD_{50} value meaningless.

To study the course of infection following oral inoculation, the strains 3 Nal^r and 3(pBL001$^-$) Nal^r (10^8 c.f.u. in 0·1 ml) were inoculated orally into two groups of 40 newly hatched chickens. At intervals thereafter three chickens from each group were killed. Visceral organs were sampled and bacteriological analysis was done using the method of Barrow *et al.* (1987*a*).

Strains 3 and 3(pBL001$^-$) were tested for their ability to survive and grow in the internal organs of the young chicken. Groups of 24 2-d-old chickens were inoculated with 10^5 c.f.u. in 0·05 ml into one of the gastrocnemius muscles. At 3 d intervals post-inoculation three chickens from each group were killed. Viable counts of the inoculated organisms in the liver, spleen, heart blood, heart muscle and both the inoculated and the corresponding uninoculated gastrocnemius muscle were made using MacConkey agar (Miles & Misra, 1938).

Growth of bacteria in the yolk sac. The method has been described previously (Barrow *et al.*, 1987*a*). Briefly, newly hatched chickens were inoculated directly into the yolk sac, through the body wall, with approximately 10^3 organisms in 0·1 ml Dulbecco's phosphate buffered saline (PBS, Oxoid BR14a). The chickens were killed 24 h later. The yolk sacs were removed and viable counts were made on their contents (Miles & Misra, 1938).

Growth of bacteria in serum and broth. Overnight broth cultures were diluted 1 in 100 in nutrient broth and further incubated with shaking at 37 °C for 2 h. The cultures were centrifuged at 1500 *g* for 30 min and the cells resuspended in an equal volume of PBS. The suspension was diluted 20-fold with PBS and 0·03 ml of this suspension was mixed with 1·5 ml of nutrient broth, of normal rabbit serum or of pooled serum obtained from healthy 4-d-old chickens. In both cases the serum was prepared by allowing fresh aseptically drawn blood to clot for 2 h at 37 °C followed by further incubation at 4 °C for 1 h before the serum was pipetted off and used immediately. The mixtures of bacteria and serum or broth were incubated statically at 37 °C. Viable counts were done on MacConkey agar at intervals thereafter (Miles & Misra, 1938).

RESULTS

Plasmid content of S. pullorum

The plasmid profiles of eight epidemiologically separate strains of *S. pullorum* are shown in Fig. 1 (lanes 1–8). Seven of these strains contained one large plasmid, in four cases the plasmids were approximately 85 kb in size (lanes 1–4) and in three, slightly larger (lanes 5–7). One strain possessed two large plasmids (lane 8). In addition, each of the eight strains possessed at least one smaller plasmid of between 2·5 and 4 kb.

One of the strains, *S. pullorum* strain 3, was chosen for further study because it possessed one large plasmid only, of 85 kb, designated pBL001 (Fig. 1, lane 1), and because some of the virulence characteristics of this strain had already been described (Smith & Tucker, 1980). Transposon Tn*3*, which confers ampicillin resistance, was used to 'tag' pBL001, which was then cured by passage of strain 3(Tn*3*) in broth containing acridine orange. The plasmid was cured at a frequency of approximately 10^{-4}. The pBL001-cured derivative, designated 3(pBL001$^-$), was

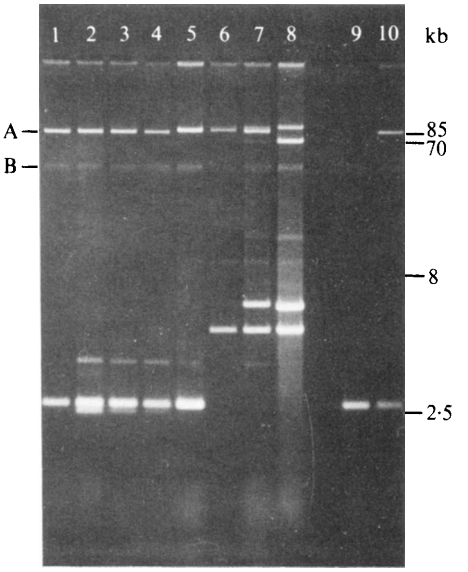

Fig. 1. Electrophoresis of *S. pullorum* plasmid DNA. Lane 1, *S. pullorum* strain 3; lanes 2–8, recently isolated field strains of *S. pullorum*; lane 9, strain 3(pBL001$^-$); lane 10, strain 3(Tn*3*)Nal^r. A shows the position of the large plasmids and B shows the position of chromosomal DNA. Additional smaller plasmids are also visible. Other faint bands in tracks represent multimeric forms of plasmids or plasmids with single strand 'nicks'. The positions of size markers are indicated on the right.

identified by its sensitivity to ampicillin and the absence of pBL001, confirmed by plasmid content analysis (Fig. 1, lane 9).

Plasmid pBL001 was reintroduced into a Nal^r mutant of the pBL001-cured derivative, designated 3(pBL001$^-$) Nal^r, by a three-factor mating using the F plasmid for mobilization (Fig. 1, lane 10). The absence of the F plasmid from the final recipient, strain 3(Tn*3*) Nal^r, was demonstrated by its resistance to the F-specific bacteriophage MS2. Both strains 3 and 3(pBL001$^-$) were found to possess smooth lipopolysaccharide by absence of agglutination with acriflavine and both reacted with O-specific antisera. No association was observed between the possession of pBL001 and either carbohydrate fermentation or standard biochemical tests used for the identification of the *Enterobacteriaceae* as determined by the API 20E and 50CH systems (API). Strains 3 and 3(pBL001$^-$) were both serum resistant in normal rabbit serum. The growth rates of the two strains in serum obtained from healthy 4-d-old chickens and in nutrient broth were similar to each other.

We were unable to 'tag' the smaller molecular mass plasmid in strain 3 despite having examined 45 Amp^r Tet^s cultures for an increase in molecular mass of this plasmid, which would have been detectable after Tn*3* insertion.

Association of pBL001 with virulence

The virulence of *S. pullorum* strain 3 and its derivatives was assessed by oral and intramuscular inoculation of newly hatched RIR chickens (Table 2). By oral inoculation, strains 3 and 3(Tn*3*) produced mortality figures that were not significantly different from each other. Mortality commenced 11 and 10 d, respectively, post-inoculation. In both cases the inoculated organisms were regularly re-isolated from the livers of chickens which had died. The clinical signs of the disease produced by both strains were similar. These included lethargy, anorexia, caked vents and the presence of yellow urates in the droppings. Yellowish spot-like areas of necrosis, 1–2 mm in diameter, were seen in the liver, heart and spleen of the dead chickens. By contrast, strain 3(pBL001$^-$) killed none of the 51 chickens inoculated orally. This was significantly different ($P < 0{\cdot}01$) from the mortality rate produced by strain 3. There were no

Table 2. *Percentage mortality and LD_{50} values of S. pullorum strain 3 and its derivatives in newly-hatched chickens*

Strain	Mortality: No of chickens inoculated orally*	Mortality: No. dead†	Mortality: Significance‡ χ^2	Mortality: Significance‡ P	LD_{50}: $\log_{10}$ C.f.u.§	LD_{50}: Significance‡ χ^2	LD_{50}: Significance‡ P
3	34	24 (71)	—	—	3·38 ± 0·43	—	—
					4·62 ± 0·33		
3(Tn*3*)	26	23 (88)	2·76	0·1	3·50 ± 0·31	0·46	0·50
					ND	—	—
3(pBL001⁻)	51	0 (0)	13·6	<0·01	5·54 ± 0·28	13·6	<0·01
					5·94 ± 0·27	16·27	<0·01
3(Tn*3*) Nal^r	34	21 (62)‖	0·59	0·4	3·78 ± 0·25	0·66	0·50
					4·38 ± 0·29	0·04	0·85

ND, Not done.

* All inoculations contained 10^8 c.f.u. in 0·1 ml.

† The percentage of the total number of chickens is given in parentheses.

‡ Results compared for statistical significance with values for strain 3.

§ Chickens inoculated intramuscularly (gastrocnemius muscle) with decimal dilutions of broth cultures, 5 chickens per dilution. Values are given for two replicate experiments and are the means ± SEM.

‖ Mortality produced by one of five colonies of strain 3(Tn*3*) Nal^r tested.

signs of ill health and strain 3(pBL001⁻) was not re-isolated from the liver when the chickens were killed four weeks later. Plasmid pBL001 was reintroduced into a single clone of strain 3(pBL001⁻) Nal^r in five separate mating experiments. In each case the virulence of the final recipient, strain 3(Tn*3*) Nal^r, was tested by oral inoculation of groups of 34 chickens. The percentage mortalities produced by these five independently derived transconjugants with the significance in parentheses of the difference between the mortality value and that produced by strain 3, were 62 ($P = 0·4$), 62 ($P = 0·4$), 56 ($P = 0·2$), 50 ($P = 0·07$) and 35 ($P < 0·01$) respectively. Thus, three of the five transconjugants with pBL001 were clearly as virulent as the parent strain. In chickens which became ill and died, clinical signs were similar to those in chickens inoculated with strain 3. Chickens began to die 9 d post-inoculation.

The LD_{50} values following intramuscular inoculation of strains 3(Tn*3*) and 3(Tn*3*) Nal^r were not significantly different from those of strain 3. This was also the case when strains 3 and 3(Tn*3*) Nal^r were re-tested on another occasion. The LD_{50} values of strain 3(pBL001⁻) were significantly different ($P < 0·01$) from those of strain 3. On one occasion the LD_{50} was increased by a factor of 150 and on another, by a factor of 12.

Strain 3 and 3(pBL001⁻) were tested for intramuscular virulence in newly hatched chickens and in 2- and 4-d-old chickens. By intramuscular inoculation the LD_{50} values of strain 3 at these ages were ($\log_{10}$ c.f.u., mean ± SEM) 4·03 ± 0·38, 6·10 ± 0·25 and 7·37 ± 0·23, respectively. The corresponding values for strain 3(pBL001⁻) were 5·55 ± 0·31, 7·63 ± 0·27 and 8·25 ± 0·21, respectively. At each age of inoculation the LD_{50} value of strain 3 was significantly different from that of strain 3(pBL001⁻). The significance values were $P < 0·01$ at 0 and 2 d and $P = 0·03$ at 4 d of age.

The course of the infection produced by strains 3 Nal^r and 3(pBL001⁻) Nal^r

The course of infection was followed after oral inoculation of newly hatched chickens with strains 3 Nal^r and 3(pBL001⁻) Nal^r. The results are summarized in Table 3. Soon after inoculation strain 3 Nal^r was isolated from the three sections of the alimentary tract and in high numbers from the contents of the caecum. By 2 d post-inoculation this strain was present in the liver and spleen and by 4 d these organisms were also present in the kidney, heart muscle and yolk sac. After 6 d the strain was isolated only intermittently from the alimentary tract, but was still present in the other tissues with the exception of blood and breast muscle. The strain persisted in the liver, spleen and heart throughout the experiment. Isolations were made from

Table 3. *Isolation of mutants of S. pullorum strains 3 Nal^r and 3(pBL001$^-$) Nal^r from the viscera of newly hatched chickens at intervals after oral inoculation*

All chickens were inoculated orally with 10^8 c.f.u. in 0·1 ml. Viable counts are expressed as median values from three chickens. Deaths occurred from 10 d post-inoculation with *S. pullorum* 3 Nal^r.

		$\log_{10}$ [C.f.u. (g tissue)$^{-1}$]									
		Days post-inoculation:									
Strain	Organ/tissue	¼	½	1	2	4	6	8	10	17	24
3 Nal^r*	Blood	N	N	N	N	N	N	N	2·0	N	N
	Kidney	N	N	N	N	2·9	2·5	4·4	3·5	N	N
	Heart muscle	N	N	N	N	3·5	4·6	5·0	5·0	5·2	5·4
	Liver	N	N	N	3·0	4·5	3·7	4·7	3·5	2·0	2·0
	Spleen	N	N	N	2·0	4·8	3·3	5·0	3·6	2·8	4·0
	Yolk sac	N	N	N	N	2·0	6·5	6·7	8·4	6·4	6·5
	Crop contents	5·0	5·2	4·7	3·3	3·7	N	3·3	N	N	2·7
	Jejunum contents	4·3	3·3	N	N	N	N	N	N	N	N
	Caecal contents	7·9	9·5	9·0	8·6	4·9	N	3·7	N	6·8	5·3
3(pBL001$^-$) Nal^r†	Liver	N	N	N	N	N	N	N	N	N	N
	Spleen	N	N	N	N	N	N	2·6	N	N	N
	Crop contents	5·2	5·3	3·6	2·6	N	N	N	N	N	N
	Jejunum contents	4·2	2·5	N	N	N	N	N	N	N	N
	Caecal contents	8·2	9·5	9·6	8·9	2·5	N	N	N	N	N

* Counts for breast muscle were zero at all times.
† Counts for blood, breast muscle, kidney, heart muscle, yolk sac and jejunal contents were zero at all times.
N, Median count ($\log_{10}$) < 2·0.

the blood at 10 d, by which time chickens usually started to die. At this time, whitish spot-like areas of necrosis were also noticed in the heart. The numbers of organisms in the yolk sac increased to a maximum at 10 d and then remained at slightly lower levels thereafter. By contrast, strain 3(pBL001$^-$) Nal^r was isolated infrequently from the spleen and was not isolated from the liver, heart or other organs. Although, as with strain 3 Nal^r, strain 3(pBL001$^-$) Nal^r was found in high numbers in the alimentary tract soon after inoculation when the indigenous gut flora is very rudimentary, it subsequently disappeared from the gut and was not re-isolated.

Survival of strains 3 and 3(pBL001$^-$) in the tissues following intramuscular inoculation

The recovery of strains 3 and 3(pBL001$^-$) from various tissues after inoculation into one of the gastrocnemius muscles of 2-d-old chickens is shown in Table 4. At 2 h post-inoculation the highest numbers of organisms of both strains were found in the inoculated muscle and the spleen. At this time, strain 3(pBL001$^-$) but not strain 3 was also isolated from the liver and heart blood in lower numbers.

The chickens inoculated with strain 3 increasingly showed signs of disease, with deaths occurring from 18 d post-inoculation. The strain was isolated from the liver and in increasing numbers from the spleen of these chickens and white areas of necrosis appeared on the surface of both organs. Liver lesions were small and localized, while spleen lesions were generally larger and more diffuse. Bacteria persisted in the inoculated gastrocnemius muscle throughout the experiment and localized abscesses were observed in the muscle. These had disappeared by 18 d. Organisms were isolated in increasing numbers from the heart muscle, but were not isolated either from cardiac blood or from the uninoculated gastrocnemius muscle. By day nine small lesions were visible in the heart muscle. Later in the experiment the lesions had enlarged until two or three lesions accounted for 20–60% of the surface of the heart. The pericardium did not appear to be affected although pericardial fluid was not cultured.

Strain 3(pBL001$^-$) was only isolated from the spleen and inoculated muscle and in decreasing numbers until it disappeared from these tissues by 24 d and 15 d respectively. The chickens in this group remained healthy throughout and the organs appeared macroscopically normal.

Table 4. *Isolation of S. pullorum strains 3 and 3(pBL001⁻) from the viscera of 2-d-old chickens after intramuscular inoculation*

All chickens were inoculated into one gastrocnemius muscle with 10^5 c.f.u. in 0·05 ml. Viable counts are expressed as median values from three chickens. Deaths occurred from 18 d post-inoculation with strain 3.

		$\log_{10}$ [C.f.u. (g tissue)$^{-1}$]								
		Days post-inoculation:								
Strain	Organ/tissue	$\frac{1}{12}$	3	6	9	12	15	18	24	30
3	Liver	N	4·2	3·8	3·3*	3·3*	4·2*	N*	2·0*	N
	Spleen	4·1	4·5	4·7*	3·6	4·4*	4·8*	3·2*	3·1	3·8
	Heart muscle	N	2·3	3·1	3·5*	4·8*	3·7*	4·0*	N*	4·5*
	Heart blood	N	N	N	N	N	N	N	N	N
	Inoculated muscle	5·2	5·2	5·8	5·0	4·7	6·0	4·8	2·9	2·5
	Uninoculated muscle	N	N	N	N	N	N	N	N	N
3(pBL001⁻)	Liver	2·0	N	N	N	N	N	N	N	N
	Spleen	4·5	5·0	4·2	4·1	2·6	2·9	2·0	N	N
	Heart muscle	N	N	N	N	N	N	N	N	N
	Heart blood	2·0	N	N	N	N	N	N	N	N
	Inoculated muscle	4·5	5·2	4·5	4·6	3·5	N	N	N	N
	Uninoculated muscle	N	N	N	N	N	N	N	N	N

N, Median count ($\log_{10}$) < 2·0.
* Macroscopic lesions observed.

Growth of bacteria in the yolk sac

Viable counts of strains 3 and 3(pBL001⁻) in the yolk sacs of two groups of five newly hatched chickens were made 24 h post-inoculation with 10^3 organisms. The median viable count of strain 3 ($\log_{10}$ c.f.u.) with the range in parentheses was 6·4 (3·2–8·4) and that of strain 3(pBL001⁻) was 6·0 (4·6–8·4).

DISCUSSION

Associations between virulence in *Salmonella* serotypes that routinely produce invasive systemic disease and the possession of large molecular mass plasmids have been reported by Jones *et al.* (1982); Terakado *et al.* (1983); Popoff *et al.* (1984); Baird *et al.* (1985); Helmuth *et al.* (1985); Nakamura *et al.* (1985) and Barrow *et al.* (1987*b*). However, in order to demonstrate the association between the possession of a plasmid and a virulence characteristic it must be shown not only that curing the plasmid is associated with loss of virulence, but that virulence can be restored by reintroduction of the plasmid. This is particularly true where relatively harsh chemical methods are used for plasmid curing, methods which might conceivably affect other non-plasmid mediated functions.

In the present study elimination of the large plasmid (pBL001) of a *S. pullorum* strain was associated with complete loss of virulence when inoculated orally, the natural route of infection. By the parenteral route there was a decrease in virulence by 150-fold on one occasion and 12-fold on another, which is, however, a considerably smaller reduction in virulence than that seen following inoculation with *S. gallinarum* and other *Salmonella* serotypes. Reintroduction of pBL001 restored oral and parenteral virulence, thereby fulfilling the above criterion for a causal association. This criterion was, however, only fulfilled by testing the virulence of transconjugants [strain 3(Tn*3*) Nal^r] which had resulted from several independent mating experiments to restore pBL001 to strain 3(pBL001⁻) Nal^r. Some of the recipients were of significantly reduced virulence. This was possibly the result of preferential conjugation between the donor strain, 3(Tn*3*) Spc^r, and individual organisms of strain 3(pBL001⁻) Nal^r which were mutants of reduced virulence (Smith, 1972; Smith & Tucker, 1979). Since at least three of the clones tested were of comparable virulence to the parental strain 3 it seemed unlikely that in these cases the acridine orange treatment used in curing had produced a significant reduction in

virulence other than that associated with plasmid loss. At this stage, and without more detailed plasmid analysis, it is impossible to say whether the large plasmids of the other strains of *S. pullorum* examined were also involved in virulence. In addition to 85 kb plasmids both *S. gallinarum* (Barrow *et al.*, 1987*b*) and *S. pullorum* possess small plasmids. Whereas the 2·5 kb plasmid possessed by *S. gallinarum* strain 9 did not contribute to virulence, the role, if any, of that in the *S. pullorum* strain studied could not be established herein as we were unable to obtain Tn*3* insertions in the small plasmid of strain 3.

Studies on the pathogenesis of the infection produced by the strains 3 Nal^r and 3(pBL001$^-$) Nal^r revealed that pBL001 was associated with more than one stage of the pathogenesis as was also observed with *S. gallinarum* strain 9 (Barrow *et al.*, 1987*b*). Its presence was associated with invasiveness, since following oral inoculation strain 3 Nal^r was isolated from the liver and spleen after a shorter period of time than was strain 3(pBL001$^-$) Nal^r, despite the presence of high numbers of both strains in the alimentary tract. Whether loss of pBL001 resulted in reduced invasiveness *per se* or in reduced translocation of organisms between the gut and the reticuloendothelial system as suggested by Gulig & Curtiss (1987) for *S. typhimurium* is as yet unclear. Results showed that the chickens became considerably more resistant to intramuscular inoculation after a few days. Thus, slower invasion or translocation must consequently lead to a reduction in the severity of the infection. These results are in contrast to those relating to *S. dublin* (Manning *et al.*, 1986) and *S. typhimurium* (Hackett *et al.*, 1986; Pardon *et al.*, 1986) in which invasion is thought to be chromosomally mediated.

Strain 3 Nal^r, but not strain 3(pBL001$^-$) Nal^r, was isolated in considerable numbers from the yolk sac following oral inoculation. Since strains 3 and 3(pBL001$^-$) Nal^r both grew well *in vivo* in the yolk sac following direct inoculation into this organ it is likely that the isolation of only strain 3 Nal^r from the yolk sac following oral inoculation can also be attributed to its greater invasiveness. Similar observations were made with strains of *S. typhimurium* of different degrees of invasiveness (Barrow *et al.*, 1987*a*).

The possession of pBL001 was also associated with increased ability to grow in tissues. Despite the absence of an association between pBL001 and serum resistance, strain 3 persisted in the inoculated gastrocnemius muscle for a much longer period than did strain 3(pBL001$^-$). The reason for the particular localization in the myocardium following intra-muscular and oral inoculation is unclear, particularly since the strain did not localize in uninoculated gastrocnemius muscle.

Salmonella in general is thought to be an intracellular pathogen (Suter & Ramseier, 1964; Takeuchi, 1967; Takeuchi & Sprinz, 1967; Popiel & Turnbull, 1985). The inability to culture *Salmonella* from either the heart blood or uninoculated muscle tends to support this contention. Serum resistance *per se* is, therefore, unlikely to be of major importance as a virulence characteristic. The large plasmid of *S. gallinarum* strain 9 was not involved in serum resistance (Barrow *et al.*, 1987*b*). Similarly plasmid pBL001 appeared to contribute little to this characteristic in *S. pullorum* strain 3. This agrees with some observations with *S. typhimurium* (Pardon *et al.*, 1986; Gulig & Curtiss, 1987), but contrasts with other findings on that serotype (Jones *et al.*, 1982; Hackett *et al.*, 1986; 1987; Vandenbosch *et al.*, 1987). It is unclear at this stage whether some authors have examined strains which possessed unidentified chromosomal lesions or whether in some cases serum resistance might be involved in virulence. Even if serum resistance is not directly involved in the pathogenesis of salmonellosis it may conceivably act as a marker for some other virulence characteristic in *S. typhimurium*.

Barrow *et al.* (1987*b*) showed by Southern hybridization that there was considerable homology between the 85 kb plasmid of *S. pullorum* strain 3 and the 85 kb plasmid of *S. gallinarum* strain 9. Similarities between these two strains in plasmid-mediated invasiveness and ability to grow in the tissues would thus not be surprising. Further studies are now under way to define the relationship between the plasmid-mediated functions in *S. pullorum* and *S. gallinarum*.

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