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Contribution of *Salmonella gallinarum* Large Plasmid toward Virulence in Fowl Typhoid

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Four strains of *Salmonella gallinarum* isolated from independent cases of fowl typhoid all possessed both an 85-kilobase and a 2.5-kilobase plasmid. Each plasmid was eliminated in turn from one of the strains by transposon labeling and curing at 42°C. Elimination of the small plasmid had no effect on the high virulence of the strain for newly hatched and 2-week-old chickens. Whereas oral inoculation of 2-week-old chickens with the parent strain produced 90% mortality with characteristic signs of fowl typhoid, inoculation of the large-plasmid-minus strain produced 0% mortality. A corresponding increase in the 50% lethal dose from $\log_{10}$ 1.1 to greater than $\log_{10}$ 7.3 was seen with the large-plasmid-minus strain after intramuscular inoculation. Reintroduction of the large plasmid completely restored virulence. A role for the plasmid-linked virulence genes in both invasion and growth in the reticuloendothelial system is suggested by the failure of the large-plasmid-minus strain to penetrate to the liver and spleen after oral inoculation and by its increased clearance from the reticuloendothelial system after intravenous inoculation. These results clearly demonstrate that the large plasmid of *S. gallinarum* contributes toward virulence in fowl typhoid of chickens.

There is increasing evidence of the involvement of plasmid-linked genes in the virulence of enteroinvasive organisms such as *Shigella* spp. and *Escherichia coli* (5). Large plasmids of various sizes have been found in several serotypes of *Salmonella* all of which produce a systemic type of disease in animals or humans. These include *Salmonella typhimurium*, *S. dublin*, *S. enteritidis*, *S. cholerae-suis*, *S. paratyphi-C*, and *S. abortus-ovis* (1, 8, 9, 13, 14, 17). The role of a 60-megadalton plasmid in the virulence of *S. typhimurium* was demonstrated by Jones et al. (9), 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 mice and possession of an 80-kilobase (kb) plasmid by *S. dublin* was demonstrated by Chikami et al. (4) using 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 (16).

S. gallinarum produces fowl typhoid of poultry (15), which is a disease of major economic importance in many countries (6). Unlike the closely related avian pathogen *S. pullorum*, *S. gallinarum* is able to produce disease in both young and adult chickens. Mortality associated with the disease is frequently high; under experimental conditions with newly hatched chickens it can be 100% (16).

This paper reports an association between the presence of a plasmid of approximately 85 kb in *S. gallinarum* and the ability of strains to produce high mortality in chickens. A relationship between this plasmid and those from other invasive serotypes of *Salmonella* is also demonstrated.

MATERIALS AND METHODS

Bacterial strains. The four strains of *S. gallinarum* used in this study were isolated from field cases of fowl typhoid occurring at different sites, and along with the other serotypes used were maintained on Dorset egg slopes at 4°C *S. pullorum* 3 and *S. typhimurium* Bangor have both been shown to possess various degrees of virulence for newly hatched chickens (16). *S. dublin* 188 is a strain virulent for calves. *S. enteritidis* 326 was also included in the study. All strains have only a large plasmid except *S. pullorum* which also contains a small plasmid. Unless otherwise indicated, L broth or L agar was used for bacterial cultivation. Broth cultures of *S. gallinarum* 9 and its three derivatives were incubated for 24 h at 37°C in a shaking water bath, and all contained approximately 1.8×10^9 viable organisms per ml.

Plasmid isolation. For preliminary visualization of plasmids the method of Kado and Liu (10) was used, while for the isolation of purified plasmid DNA the method of Hansen and Olsen (7) was used. Electrophoresis of plasmid DNA was carried out with 0.7% agarose gels. The size of the large plasmid was estimated by direct comparison with the 60-megadalton plasmid of *S. typhimurium* and the 50-megadalton plasmid of *S. dublin* 188. The size of the small plasmid was estimated after it was linearized with *Hin*dIII and compared with λ *Hin*dIII markers. Southern blots were done as previously described (12), and filters were probed with plasmid DNA which had been labeled with [^{32}P]dCTP by nick translation (12).

Transposon mutagenesis and plasmid curing. To facilitate curing, we tagged the plasmids present in *S. gallinarum* 9 with the transposon Tn*3*. This was achieved by using the temperature-sensitive transposon donor plasmid pMB501 as previously described (3). The donor plasmid was introduced into *S. gallinarum* by transformation (11). For mutagenesis, a colony containing pMB501 was grown at 42°C for 15 h. Bacteria were then streaked onto L agar plates containing ampicillin (100 μg/ml) which were incubated for a further 15 h at 42°C. Loss of the donor plasmid was confirmed by screening colonies for their sensitivity to tetracycline, resistance to which is encoded by pMB501. The insertion of the transposon into the large or small plasmid was verified by plasmid analysis.

Tagged plasmids were cured by growth at 42°C for 18 h. Plasmid loss was initially identified by sensitivity to ampicillin and confirmed by plasmid analysis. Loss of the small plasmid under these conditions was relatively common,

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occurring in approximately 1% of cells. The frequency of curing of the large plasmid was, however, much lower, occurring in less than 0.1% of cells.

Bacterial mating. To reintroduce the large plasmid into the strain from which it had been cured, we used a three-factor mating. The initial donor was *E. coli* K-12 F^+, the intermediate was a rifampin-resistant mutant of *S. gallinarum* 9 containing the large plasmid tagged with Tn*3*, and the final recipient was a nalidixic acid-resistant mutant of *S. gallinarum* 9 from which the large plasmid had been cured. Mating was carried out at 37°C for 18 h followed by a further 8 h at room temperature, the mating mixture being plated out on L agar containing ampicillin (100 μg/ml) and nalidixic acid (20 μg/ml). Colonies were checked for rifampin sensitivity to ensure that they were not spontaneous nalidixic acid-resistant mutants of the intermediate.

Virulence tests. Newly hatched and 2-week-old Light Sussex chickens were used for oral and intramuscular virulence estimations. Briefly, undiluted broth cultures were inoculated orally into groups of 40 chickens, and 0.1-ml serial dilutions in nutrient broth were inoculated into the gastrocnemius muscle of groups of 10 chickens. For the intramuscular inoculations the dilution range used was 10^{-5} to 10^{-8} for all the strains except the large-plasmid-minus strain for which the range was undiluted to 10^{-3}. The number of deaths over a 2-week period was recorded, and the percent mortality and 50% lethal dose (LD_{50}) were recorded.

For estimation of bacterial invasion, spontaneous mutants of the parent and the large-plasmid-minus strains resistant to nalidixic acid (20 μg/ml) were isolated. Groups of 15 newly hatched and 15 2-week-old chickens were inoculated orally with 0.1 ml of undiluted overnight broth cultures of these two strains. At intervals after inoculation three birds from each group were killed, and viable counts were estimated for the liver, spleen, and cecal contents on MacConkey agar containing nalidixic acid (20 μg/ml) and novobiocin (1 μg/ml).

To assess viability in the reticuloendothelial system, two groups of 18 2-week-old chickens were inoculated intravenously with 0.1 ml of a 10^{-2} dilution (parent strain) or a 10^{-1} dilution (large-plasmid-minus strain) of overnight broth cultures of the antibiotic-sensitive forms of the two strains. At intervals three chickens from each group were killed, and viable counts were estimated for samples of cardiac blood, liver, and spleen. The first sample, 0 days, was taken 1 h after inoculation.

Serum resistance tests were carried out as described previously (2). Briefly, overnight broth cultures of the strains were diluted 1/50 in L broth and grown with shaking at 37°C. Cells from exponentially growing cultures at an optical density at 600 nm = 0.4 were collected by centrifugation and suspended in phosphate-buffered saline at an optical density at 600 nm = 0.44. The suspension was then diluted 100-fold with phosphate-buffered saline, and 0.1 ml of these cells was then mixed with 0.6 ml of normal rabbit serum. The suspension of bacteria in serum was incubated at 37°C without shaking, and viable counts were made at 0, 75, 135, and 195 min.

RESULTS

Plasmid content of *S. gallinarum* strains. The presence of serotype-specific plasmids in a number of *Salmonella* strains has been described previously (1, 8, 9, 13, 14, 17). Examination of the plasmid content of four *S. gallinarum* strains from separate disease outbreaks revealed that the plasmid profiles were identical. All strains contained one large plasmid of approximately 85 kb and one small plasmid of 2.5 kb (Fig. 1).

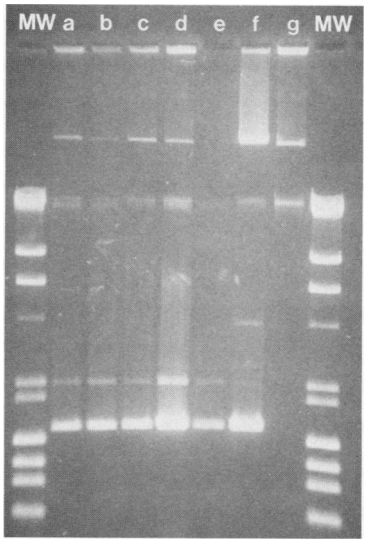

FIG. 1. Plasmid profiles of *S. gallinarum* strains. Lanes: a, *S. gallinarum* 149; b, *S. gallinarum* 7285; c, *S. gallinarum* Blandford; d, *S. gallinarum* 9; e, *S. gallinarum* 9 large plasmid cured; f, same as lane e with the large plasmid reintroduced; g, *S. gallinarum* 9 small plasmid cured. Other bands on the gel represent alternative forms of the small plasmid and chromosomal DNA. Lanes MW are λ *Hin*dIII and φX174 *Hae*III fragments.

To assess the contribution of these plasmids to the virulence of *S. gallinarum*, we constructed mutants of strain 9 which were cured of each plasmid. This was achieved by tagging each plasmid in turn with the transposon Tn*3* and curing by passage at 42°C. Strains cured of each plasmid were identified by their sensitivity to ampicillin, resistance to which is encoded by Tn*3*. The large plasmid was reintroduced into the strain from which it had been cured by a three-factor mating with the F plasmid. The absence of the F plasmid from the final recipient was demonstrated by its resistance to the F-specific bacteriophage MS2. This was also confirmed by examination of its plasmid content. The plasmid content of the cured strains and the strain into which the large plasmid was reintroduced are shown in Fig. 1.

Association of plasmids with virulence. The virulence of *S. gallinarum* 9 and its derivatives was assessed by oral and intramuscular inoculation of newly hatched and 2-week-old Light Sussex chickens. The number of deaths over a 2-week period was recorded, and the results are presented in Table 1.

In newly hatched chickens oral inoculation with the parent strain produced 100% mortality by 6 days after inoculation. The strain was regularly reisolated from the livers of birds that had died. By intramuscular inoculation, the LD_{50} of this strain was $\log_{10}$ 0.6. The elimination of the small plasmid had essentially no effect on virulence. After elimination of the large plasmid the strain produced was much less virulent. Oral inoculation produced 50% mortality by 9 days after inoculation. Nevertheless, the organism was reisolated from the livers of dead birds. By intramuscular inoculation, the LD_{50} of this strain was $\log_{10}$ 7.5.

Reintroduction of large plasmid restored full virulence. In 2-week-old chickens oral inoculation of the parent strain produced 90% mortality by 14 days after infection. The

TABLE 1. Virulence of *S. gallinarum* 9 and its derivatives for newly hatched and 2-week-old chickens

Strain used	Virulence of *S. gallinarum* strains inoculated in:			
	Newly hatched chickens		2-wk-old chickens	
	Peroral[a]	Intramuscular[b]	Peroral[a]	Intramuscular[b]
Parent strain	100	0.6	90	1.1
Small plasmid minus	100	0.9	78	0.5
Large plasmid minus	50	7.5	0	>7.3
Large plasmid reintroduced	100	0.7	98	1.3

[a] Percent mortality observed by 2 weeks after inoculation of 40 chickens with 0.1 ml of undiluted broth culture.
[b] Log_{10} LD_{50} value.

TABLE 3. Viability of *S. gallinarum* 9 and its large-plasmid-minus derivative in the liver and spleen of 3-week-old chickens

Time (days) after inoculation	Log_{10} viable counts after intravenous inoculation of:					
	S. gallinarum 9 Nal[a]			*S. gallinarum* 9 Nal (LP^-)[b]		
	Blood	Liver	Spleen	Blood	Liver	Spleen
0	<2	<2	4.2	4.0	4.1	5.7
2	2.3	4.9	5.0	<2	2.5	4.8
4	3.0	6.0	6.1	<2	<2	3.7
6	3.8	7.2	6.6	<2	<2	3.0
8		Died		<2	<2	2.3
10				<2	<2	<2

[a] Nal, Nalidixic acid resistant.
[b] LP^-, Large plasmid minus.

organism was always reisolated from the livers of birds that had died. The LD_{50} by intramuscular inoculation was log_{10} 1.1. Elimination of the small plasmid produced a small reduction in the level of mortality when assessed orally, but by intramuscular inoculation this strain was as virulent as the parent. Elimination of the large plasmid produced a strain which when inoculated orally produced no mortality. The intramuscular LD_{50} could not be calculated and was $>log_{10}$ 7.3. In all chickens inoculated by both routes with this strain the organism was never reisolated from the liver when the birds were killed at the end of the experiment. Reintroduction of the large plasmid again fully restored virulence. Heart and small intestinal lesions characteristic of the chronic stage of fowl typhoid were observed in chickens infected with the parent strain, the derivative in which the small plasmid had been cured, and the strain into which the large plasmid had been reintroduced. None were observed in the chickens inoculated with the derivative in which the large plasmid had been cured when they were killed at the end of the experiment. This demonstrated the importance of the large plasmid in the production of fowl typhoid.

Role of large plasmid in pathogenesis. To assess whether the large plasmid had a role in bacterial invasion, 1-day-old and 3-week-old chickens were inoculated orally with the parent and large-plasmid-minus strains, and the presence of bacteria in the liver and spleen was monitored. In 3-week-old chickens the parent strain persisted in the alimentary tract and was isolated from the liver and spleen by 60 h postinoculation (Table 2). In contrast, the large-plasmid-minus strain was eliminated from the alimentary tract after 24 h and was not isolated from the liver and spleen during the course of the experiment (84 h) (Table 2). In 1-day-old chicks high numbers of both strains were found in the cecal contents during the experiment, and organisms of both strains were isolated from the liver and spleen. This is consistent with the result that the large-plasmid-minus strain could still cause some mortality in 1-day-old birds when inoculated orally (Table 1).

To assess the viability of the strains in the reticuloendothelial system, both strains were inoculated intravenously into 3-week-old birds, and the presence of bacteria in cardiac blood, liver, and spleen samples was monitored. The large-plasmid-minus strain was cleared from the blood by 2 days postinoculation (Table 3). The viable counts of this strain in the liver and the spleen rapidly decreased, and the chickens remained healthy. In contrast, the counts of the parent strain increased in the blood, liver, and spleen, until by 8 days postinoculation all the birds were dead.

These results suggest that the plasmid has a role in pathogenesis both at the level of survival in and associated penetration of the alimentary tract and in survival and multiplication in cells of the reticuloendothelial system.

The resistance to serum of *S. gallinarum* 9 and its derivatives was assessed (Table 4). Plasmid carriage was found not to affect bacterial survival in serum, i.e., all strains were able to grow in 86% normal rabbit serum. Similarly, no association was observed between possession of plasmids and carbohydrate fermentation as determined with the API system (API Systems, Montalieu-Vercieu, France). All strains were smooth by nonagglutination with acriflavin, and all reacted with O-specific antisera.

Homology of large plasmid with other *Salmonella* plasmids. It has previously been reported that the large plasmids of a number of *Salmonella* serotypes share homology as demonstrated in Southern blots (14). To determine whether the

TABLE 2. Intestinal invasiveness of *S. gallinarum* 9 and its large-plasmid-minus derivative in newly hatched and 3-week-old chickens

Age of birds (days)	Time (h) after inoculation	Log_{10} viable counts[a] after oral inoculation with:					
		S. gallinarum 9 Nal			*S. gallinarum* 9 Nal (LP^-)[b]		
		Liver	Spleen	Ceca	Liver	Spleen	Ceca
21	12	<2	<2	5.0	<2	<2	5.0
	24	<2	<2	5.0	<2	<2	<2
	36	<2	<2	<2	<2	<2	<2
	60	3.9	4.3	3.7	<2	<2	<2
	84	ND[c]	ND	ND	<2	<2	<2
1	3	<2	<2.7	6.5	<2	<2.7	6.5
	6	<2	<2.7	8.1	<2	<2.7	8.4
	12	<2	<2.7	8.9	<2	<2.7	7.9
	24	3.0	<2.7	9.6	2.2	<2.7	9.1
	48	4.0	5.0	6.9	4.8	6.4	6.2

[a] Median count from three birds.
[b] LP^-, Large plasmid minus. Nal, Nalidixic acid resistant.
[c] ND, Not determined.

TABLE 4. Survival in serum of *S. gallinarum* 9 and its derivatives

Strain	Log_{10} viable count at time (min):			
	0	75	135	195
Parent	5.7	6.4	6.6	6.7
Small plasmid minus	5.8	6.3	6.6	6.7
Large plasmid minus	5.8	6.5	6.7	6.7
Large plasmid reintroduced	5.8	6.3	6.6	6.6

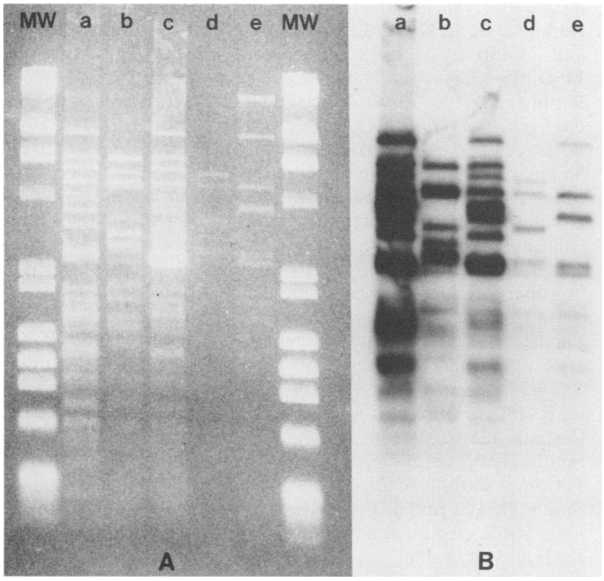

FIG. 2. (A) *Pvu*II fragments from the plasmids of the following strains: lane a, *S. gallinarum* 9 small plasmid cured; lane b, *S. typhimurium* Bangor; lane c, *S. pullorum* 3; lane d, *S. enteritidis* 326; lane e, *S. dublin* 188. Molecular weight standards (lanes MW) are the same as described in the legend to Fig. 1. (B) Autoradiograph of a Southern blot of the gel in panel A which was probed with ^{32}P-labeled plasmid from the *S. gallinarum* strain containing only the 85-kb plasmid. Extensive homology is evident between the plasmids of all the strains.

large plasmid in *S. gallinarum* is also related in this way, we isolated plasmids from a number of *Salmonella* strains in which large plasmids had been implicated in virulence. The plasmids were digested with *Pvu*II, and the fragments were separated on 1% agarose gels. After Southern blotting, the filter was probed with the large plasmid from *S. gallinarum* which had been radiolabeled with [^{32}P]dCTP. After hybridization overnight at 65°C, the nitrocellulose filter was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C for three periods of 20 min each. The results show that there is extensive homology between all the plasmids (Fig. 2). This raises the possibility that some virulence determinants are shared between all the serogroups even though the strains have different host ranges.

DISCUSSION

The results presented here clearly demonstrate the important contribution that the large plasmid makes to the virulence of *S. gallinarum* and to the production of fowl typhoid. Although no role could be ascribed to the small plasmid, it is of interest that it was possessed by all four strains in this study.

The role of the large plasmid in the pathogenesis of fowl typhoid was partially identified. It appears to encode at least two separate functions. Elimination of the large plasmid resulted in a marked reduction in virulence by the intramuscular route in both 1-day-old and 2-week-old chickens, suggesting that the plasmid enables the strains to survive and grow in the cells of the reticuloendothelial system. More detailed investigations supported this contention. Whereas after intravenous inoculation of 2-week-old chickens the large-plasmid-minus strain was rapidly eliminated from the liver and spleen and was not isolated from the blood other than soon after inoculation, the numbers of the parent strain in the liver, spleen, and blood increased to the point of death. Despite the more frequent isolation of the parent strain from blood after intravenous inoculation, the plasmid did not appear to encode serum resistance, as both parent and plasmid-minus strains were capable of growth in serum.

Whereas in 2-week-old chickens elimination of the large plasmid resulted in a reduction in virulence by the oral route, this was less marked in 1-day-old chickens. This may be explained by the absence of an established gut flora in day-old birds which would allow massive microbial multiplication from small inocula. Such multiplication makes oral LD_{50} estimations in newly hatched chicks unreliable. Bacterial multiplication in the gut and subsequent invasion may account for the residual levels of killing observed with the large-plasmid-minus strain given orally to the younger birds. Experimental data indeed showed that both the parent strain and its large-plasmid-minus derivative were equally invasive in newly hatched birds. This was not so in the 2-week-old chickens in which only the parent strain was invasive. In this case the plasmid appears to mediate both survival in and penetration of the alimentary tract. In the present study, increased survival in the alimentary tract may be associated with adhesiveness. Jones et al. (9) found that adhesion in the HeLa cell test was associated with a large plasmid in *S. typhimurium* LT2. It may also be significant that in our studies the large-plasmid-minus strain isolated from the cecal contents grew more poorly than the parent strain.

Considerable homology appears to exist between the large plasmids of *S. gallinarum*, *S. pullorum*, *S. typhimurium*, *S. dublin*, and *S. enteritidis* as judged by the results of the Southern blot shown in Fig. 2. Caution must be exercised in interpreting these results as the presence of insertion sequences could give a false impression of plasmid relatedness. However, it is unlikely that insertion sequence elements would account for as many fragments sharing homology as are seen in Fig. 2. Baird et al. (1) have recently identified a virulence sequence on the large plasmid of *S. dublin* which is also present on the large plasmid present in *S. typhimurium*. Their results suggest that although different serotypes of *Salmonella* infect different host species, they share some common virulence determinants. It would be of considerable interest to determine whether the sequence they have identified is also present on the large plasmid of *S. gallinarum*.

For studying the association between virulence in *Salmonella* species and the presence of large plasmids, the present system has the obvious advantage that the disease process can be examined in the natural host species. The problems of extrapolating results from heterologous model systems are illustrated by the results of Nakamura et al. (13). These workers showed that with a strain of *S. enteritidis* isolated from cattle the presence of a 36-megadalton plasmid was associated with a virulence for mice but not for young chicks, thereby producing a confusing picture of the importance of the plasmid.

Further studies will attempt to define the plasmid genes involved in the pathogenesis of fowl typhoid by molecular cloning and mutagenesis experiments.

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