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Plasmid-Mediated Virulence in *Salmonella dublin* Demonstrated by Use of a Tn*5*-*oriT* Construct

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***Salmonella dublin*, a serotype which causes invasive disease in cattle and humans, carries a characteristic 80-kilobase plasmid (pSDL2). We were able to cure the plasmid from a strain of *S. dublin*. The cured strain was avirulent for mice by either the oral or intraperitoneal route of infection. A derivative of Tn*5* which contains the transfer origin of the broad-host-range plasmid RK2 (Tn*5*-*oriT*) was transposed onto pSDL2, allowing mobilization of the plasmid by an RK2 helper plasmid. Reintroduction of the pSDL2 derivative plasmid into the cured strain restored virulence, demonstrating that the plasmid is necessary for virulence. These studies also demonstrate the usefulness of the Tn*5*-*oriT* construct for genetic manipulations.**

Plasmids have been shown to carry genes that mediate virulence properties of several important enteric bacteria (6), including toxigenic and invasive *Escherichia coli* strains (13, 26), *Shigella* spp. (17, 23, 29), *Yersinia* spp. (22, 32), probably *Salmonella typhimurium* (14, 16) and *S. enteritidis* (14, 20) and *S. cholerasuis* (14). *S. dublin* is a serotype that is host adapted to cattle and constitutes a major veterinary problem worldwide. Infection in humans, which is relatively uncommon, is often acquired by drinking unpasteurized milk (7, 30). In both cattle and humans, *S. dublin* is an unusually invasive serotype. After oral inoculation of calves, *S.dublin* invades through the distal ileum and cecum, then spreads to the region lymph nodes and subsequently causes systemic infection (12). Patients harboring *S. dublin* frequently present with bacteremia and metastatic infection (7). Terakado et al. (27) examined 20 *S. dublin* strains from cattle and reported that a 50-megadalton plasmid was present in all 20. These workers cured the plasmid from one strain and found that the cured derivative was less virulent for mice by intraperitoneal injection. Helmuth et al. (14) also reported the association of virulence of *S. dublin* with the presence of a 56-megadalton plasmid; however, the *Hin*dIII restriction pattern of the plasmid is different from that of the plasmid reported by Terakado et al. (27).

We studied a strain of *S. dublin* isolated from the blood of a patient and found that this organism contained a plasmid of similar size and restriction pattern to the plasmid described by Terakado et al. (27), but which was different from that reported by Helmuth et al. (14). We used a derivative of the transposon Tn*5*, containing the transfer origin of RK2 (Tn*5*-*oriT* [31]), to facilitate the genetic manipulation of this *S. dublin* plasmid. Transposition of Tn*5*-*oriT* onto the *S. dublin* plasmid allowed the plasmid to be transferred between bacterial strains by an RK2 helper plasmid. These studies show that the *S. dublin* plasmid is a major determinant of virulence in mice by both oral and intraperitoneal routes of infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. dublin* Lane was isolated from the blood of a patient at the Veterans Administration Medical Center, San Diego, Calif., and was identified by standard microbiological criteria (8). *S. dublin* 178/72, obtained from B. Stocker, Stanford University, Stanford, Calif., is a bovine isolate from France. *E. coli* JA221 (*leuB* Δ*trpE5 lacY recA hsdR*$^-$ *hsdM*$^+$), and C2110 *nal*^{r} (*pol*A) have been described previously (10, 24). For triparental matings, the helper plasmid was in *E. coli* MV12, which is C600 Δ*trpEC recA* (15). Plasmids used are described in Table 1. Bacterial strains were grown in L broth (10% tryptone, 0.5% yeast extract, 0.5% NaCl) or tryptic soy broth. For nutritional selection, M9 medium containing 0.5% Casamino acids and 0.2% glucose and supplemented with 0.1 μg of nicotinamide per ml was used (19). Antibiotic selection was with 50 μg of kanamycin per ml, 20 μg of tetracycline per ml, 200 μg of penicillin per ml, or 200 μg of trimethoprim per ml.

Restriction enzymes and ligase. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories, Gaithersburg, Md. Reaction conditions were as specified by the vendor.

Plasmid analysis. Plasmid DNA was purified by the method of Currier and Nester (5). Rapid isolation of plasmid DNA for clone analysis of *Salmonella* spp. was done by a scaled-down modification of the Currier and Nester procedure. Cells from a 10-ml overnight culture were collected by centrifugation at 7,000 × *g* for 5 min, and the pellet was frozen and then resuspended in 850 μl of 10 mM Tris hydrochloride (pH 8.0)–5 mM EDTA (TE). Predigested pronase (100 μl of a 5-mg/ml solution in TE) and 50 μl of 20% sodium dodecyl sulfate were added. The cells were incubated at 37°C until they were completely lysed. The chromosomal DNA was then sheared by vortexing. Ice-cold isopropanol (1 ml) was added. The precipitated nucleic acids were suspended in 2 ml of 50 mM Tris hydroxide (pH 12.4)–20 mM EDTA–1% sodium dodecyl sulfate. After the nucleic acids were in solution, 200 μl of 2 M Tris hydrochloride (pH 7.0) and 200 μl of 5 M NaCl were added. The solution was then extracted once with phenol saturated with 3% (wt/vol) NaCl. The aqueous phase was extracted three times with diethyl ether. The DNA was precipitated by adding an equal volume of isopropanol with cooling to −70°C for 15 min. The DNA was collected by centrifugation at 12,000 × *g* for 30 min, lyophilized, and suspended in 50 μl of H_2O. The samples were treated with RNase before electrophoresis. Plasmid isolation for clone analysis of *E. coli* was done by the cleared lysate procedure (28).

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TABLE 1. Plasmids

Plasmid	Description	Reference
pSDL2	80-Kilobase-pair plasmid isolated from *S. dublin* Lane.	This work
pSD5	Derived form pSDL2 by insertion of Tn*5-oriT*. Km^r. Mobilized by the RK2 transfer system.	This work
pSD6	Derived from pSDL2 by insertion of Tn*5-oriT*. *Km*^{r}. Differs from pSD5 in location of Tn*5-oriT* insert. Mobilized by RK2 transfer system.	This work
pSD7	Result of ligation of partial *Eco*RI digestion of pSD5 and *Eco*RI digestion of pBR322. Contains the replication functions of pSD5, the Tn*5-oriT*, and all of pBR322. Km^r, Tc^r, Ap^r.	This work
pEYDG1	Derivative of pBR322 which has its *oriT* and Tc^r gene deleted. Tn*5* was inserted and the 760-base-pair *oriT* of RK2 was cloned into the *Bam*HI site of Tn*5*.	31
pBR322	Ap^r, Tc^r.	2
pRK212.1	Derived from RK2 by Mu insertion and *Hin*dIII deletion. Contains all necessary transfer regions. Am^r, Tc^r, *tra*$^+$.	9
pRK2073	Derived from pRK212.1 by inserting a *Sal*I Km^r fragment into the Tc^r gene, deleting the *Eco*RI fragment containing the *oriV* and Ap^r, and replacing it with the plasmid ColE1 as an *Eco*RI fragment. Tn*7* was then inserted into the Km^r gene. Tp^r, Sm^r, *tra*$^+$, *rep*ColE1.	31

Transformation and conjugation. Transformation of *E. coli* was done by the method of Cohen et al. (4). Plasmids containing Tn*5-oriT* were mobilized by cotransfer with a helper plasmid, pRK2073 (Table 1), which was in *E. coli* MV12. The donor strain, helper strain, and recipient were grown to late-log phase in L broth with appropriate selection. The cells were washed once in normal saline and resuspended in normal saline to their original volume, and then 1-ml volumes of each were combined and passed through a filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The filter was incubated on L agar without selection for 1 h at 37°C. The bacteria were then washed off the filter in normal saline. Serial 10-fold dilutions were made and plated on either L agar with appropriate antibiotic selection or M9 medium-Casamino acids-glucose with antibiotics.

Curing of plasmids. An overnight culture of the *S. dublin* strain carrying the plasmid marked with tetracycline resistance (Tc^r) was diluted 1:1,000 in L broth containing 50 μg of ethidium bromide per ml and 200 μg of novobiocin per ml (3, 18). Preliminary studies showed that this combination of ethidium bromide and novobiocin was just below the MIC for the organism. Visible growth occurred after 18 h. The bacteria were plated on Bochner media with 12 μg of fusaric acid per ml (1). Tetracycline-sensitive (Tc^s) colonies were examined for the presence of plasmids as described above.

Mouse infection. The *S. dublin* strains were grown overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C. They were centrifuged, washed in sterile saline, and suspended in 0.1 M $NaHCO_3$ to a density of 2×10^8 CFU/ml. The suspension was diluted 10^{-3} in 0.1 M $NaHCO_3$ just before inoculation. The inoculum size was confirmed by doing serial dilutions and colony counts.

Preliminary results indicated that (C57BL/6 × DBA/2)F_1 mice, carrying the *Ity*^{r} allele for resistance to *S. typhimurium* (21), were resistant to infection with *S. dublin* Lane. Therefore, female BALB/C mice (*Ity*^{s}), weighing 16 to 20 g each, were used in all experiments. For oral infection, 1.0 ml of the bacterial suspension was delivered by gavage directly into the stomach with a blunt 18-gauge needle. Intraperitoneal infection was induced by injecting 0.1 ml of a bacterial suspension. Five mice were housed per cage; the mice allowed free access to food and water. The orally infected mice were observed for 4 weeks, and the intraperitoneally infected mice were observed for 2 weeks.

RESULTS

Plasmids in *S. dublin* strains. The Lane strain of *S. dublin* was found to contain a single 80-kilobase plasmid, designated pSDL2. The *Hin*dIII restriction digest of this plasmid is shown in Fig. 1, lane A, and appears very similar to the plasmids from *S. dublin* studied by Terakado et al. (27). We examined 16 different *S. dublin* isolates, of both human and bovine origin, and found that they all carried a plasmid of 80 kilobases. The only strain that lacked this plasmid was a bovine isolate from France, *S. dublin* 178/72 (lane C).

Transposition of Tn*5-oriT* onto pSDL2. To facilitate the genetic manipulation of pSDL2, we used the Tn*5-oriT* transposon to label the plasmid with kanamycin resistance (Km^r) and promote transfer into various bacterial strains. This transposon is a derivative of Tn*5* which contains the transfer origin of the broad-host-range plasmid RK2 (11, 31). The vector plasmid pEYDG1, a pBR322 replicon containing Tn*5-oriT*, was mobilized into *S. dublin* Lane by using the helper plasmid pRK212.1. Separate *E. coli* JA221 strains containing pEYDG1 and pRK212.1 were mated with *S. dublin* Lane, and transconjugants were selected by plating on M9 medium containing kanamycin. Cells that had received only pEYDG1 were chosen after the colonies were screened for the Tc^r carried by pRK212.1. Mobilization of Km^r into *E. coli* C2110 (*polA*) *nal*^{r} was used to select for Tn*5-oriT* inserts into pSDL2. Since neither pEYDG1 nor the helper plasmid pRK2073 will replicate in strain C2110, only pSDL2::Tn*5-oriT* will transfer Km^r. A number of Tn*5-oriT* insertions into pSDL2 were obtained. The *Hin*dIII restriction digest of one of these insertion derivatives, designated pSD6, is shown in lane B. Three other independent Tn*5-oriT* insertions, designated pSD3, pSD4, and pSD5, were characterized and shown to be in different *Hin*dIII fragments.

Curing of pSDL2 from *S. dublin* Lane. To study the role of pSDL2 in the virulence of *S. dublin*, we cured the plasmid from the Lane strain. Initial attempts to cure the native plasmid with ethidium bromide or novobiocin were unsuccessful. Therefore, we displaced pSDL2 with a hybrid plasmid containing pBR322, which carries Tc^r and then selected for Tc^s cured derivatives by plating on the medium described by Bochner et al. (1). pSD5, a derivative of pSDL2 with Tn*5-oriT*, was partially digested with *Eco*RI, ligated to a complete *Eco*RI digest of pBR322, and transformed into *E. coli* C2110, selecting for both kanamycin and tetracycline resistance. A hybrid plasmid, pSD7, was obtained which retained Tn*5-oriT*, most of pSDL2, and pBR322. pSD7 was mobilized into *S. dublin* Lane by using pRK2073 as the helper plasmid, and a transconjugant that was Km^r and Tc^r but trimethoprim sensitive (Tp^s; therefore not harboring the helper plasmid) was selected for further study.

pSDL2 should have been lost by incompatibility, but to

ensure this the transconjugant was serially passed with Km^r and Tc^r selection. It was then cloned, and plasmid analysis showed that this strain contained only pSD7. This *S. dublin* Lane(pSD7) strain was then grown with ethidium bromide and novobiocin and plated on Bochner medium to select Tr^s cells as described in Materials and Methods. Two Tc^s clones were obtained. Plasmid analysis of the clones obtained confirmed that pSD7 had been eliminated. One of these cured derivatives, designated LD842 (lane E), was used for further studies. LD842 was motile and identical to the Lane strain in colony morphology, biochemical profile, and agglutination with group-specific antiserum.

pSD6, one of the Tn*5-oriT* insertion derivatives, was mobilized into LD842, with pRK2073 in *E. coli* MV12 as a helper plasmid. The mating frequency was 4.8×10^{-5}. The helper plasmid was usually present in the transconjugants, but since pRK2073 is unstable in *S. dublin*, clones which had lost pRK2073 could be easily isolated after growth for 20 to 25 generations with selection for only pSD6.

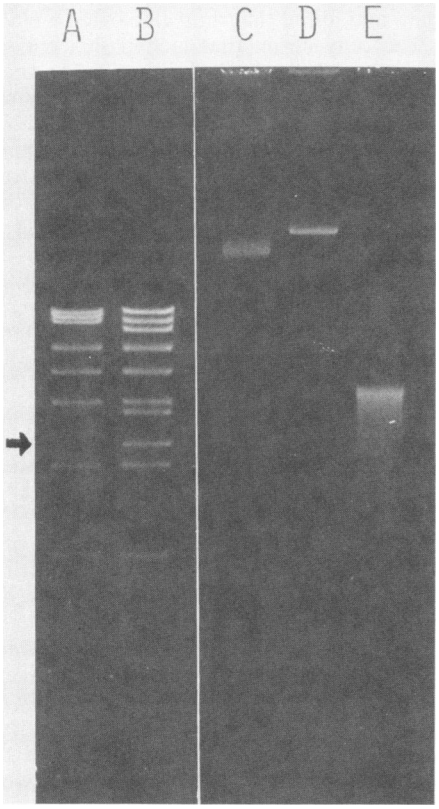

FIG. 1. Composite agarose gel electrophoresis of *S. dublin* plasmids. Lanes A and B are *Hin*dIII digests of pSDL2 and pSD6, respectively. The internal *Hin*dIII fragment of Tn*5-oriT* from pSD6 is shown by the arrow. The insertion of Tn*5-oriT* into one of the pSDL2 *Hin*dIII fragments adds two new *Hin*dIII sites. Therefore, restriction with *Hin*dIII will produce three new fragments; the internal *Hin*dIII fragment of Tn*5-oriT*, shown by the arrow, and two additional fragments which can be seen in lane B. Lanes C, D, and E represent a separate gel with unrestricted DNA: lane C, clone analysis DNA from strain 178/72; lane D, pSDL2; lane E, clone analysis DNA from strain LD842. Strain 178/72 contains a plasmid smaller than pSDL2 and a faint plasmid band considerably larger than pSDL2. Strain LD842 did not contain any detectable plasmid DNA, even when the gel was loaded with a large sample volume giving an intense, diffuse chromosomal DNA band (lane E).

TABLE 2. Virulence of *S. dublin* Lane and derivatives in mice infected by gavage

Inoculum[a]	Virulence of *S. dublin* strains[b]		
	Lane(pSDL2)	LD842	LD842(pSD6)
5×10^8	ND	0/6	ND
5×10^7	ND	0/6	ND
5×10^6	5/6	0/6	3/6
5×10^5	6/6	ND	3/6
5×10^4	3/6	ND	3/6

[a] Bacteria were delivered into the stomach as described in Materials and Methods.
[b] Number of dead mice/total number infected. ND, Not done.

Virulence studies in mice. *S. dublin* Lane was compared with the plasmid-free derivative, LD842 for virulence in mice infected by gavage (Table 2). In this model, the Lane strain was quite virulent and inocula of 5×10^4 or greater produced death in most of the animals. Mice sacrificed 4 weeks after infection all showed systemic infection with liver and spleen abscesses. In contrast, the cured strain LD842 was completely avirulent for mice by oral gavage (Table 2). Even in mice infected with the highest dose, at 4 weeks after infection there was no evidence of liver or spleen abscesses, although *S. dublin* cells could be cultured from a spleen specimen in small numbers. A strain of LD842 isolated from the spleen was reexamined for plasmid DNA and shown to remain plasmid free. The results in Table 2 also show that transfer of pSD6 into LD842 restored the virulence of the strain. Although the mortality was not quite as high as for those infected with the Lane strain, all the survivors of the infection with LD842 containing pSD6 had abscesses in the liver and spleen. The independently isolated pSD4 and pSD5 plasmids also restored the virulence of LD842 (not shown).

We examined the effect of pSD6 on the virulence of *S. dublin* 178/72, the natural bovine isolate that lacks a plasmid comparable to pSDL2. Strain 178/72 lacked virulence for mice in the oral infection model (Table 3). However, transfer of pSD6 into 178/72 increased the virulence of this strain to a level comparable to that of the Lane strain which contains pSDL2.

In addition to the oral infection model, we tested the effect of the plasmid on the virulence of *S. dublin* by intraperitoneal inoculation. The parent *S. dublin* Lane strain was highly virulent by this route of infection; all the animals died at an inoculum of 3×10^2 CFU. At the same dose, all the mice infected with strain LD842 recovered. However, LD842 containing pSD6 was as virulent as the Lane strain at the same inoculum.

TABLE 3. Increase in the virulence of *S. dublin* 178/72 by pSD6

S. dublin strain	No. of survivors/total no.[a]	No. with liver abscesses/total no.[b]
178/72	0/5	0/5
178/72(pSD6)	2/5	3/3
Lane(pSDL2)	3/5	2/2

[a] The inoculum was 6×10^6 for each strain, infected by gavage. Mice were observed for 28 days.
[b] Data given for mice alive after 28 days.

DISCUSSION

This study demonstrates that pSDL2 is required for the virulence of *S. dublin* in mice. In a recent study, Terakado et al. reported that a similar plasmid was associated with the virulence of *S. dublin* strains isolated from cattle (27). These workers found that a cured derivative of their *S. dublin* strain was less virulent by intraperitoneal inoculation in mice. However, Terakado et al. were not able to reintroduce the plasmid back into their cured strain. We found that both a natural isolate lacking the plasmid (*S. dublin* 178/72) and our cured strain, LD842, were avirulent in mice by either the oral or the intraperitoneal route. Most importantly, we were able to transfer pSD6 into 178/72 and LD842 and restore the virulence of these strains. These results show that the avirulence of strains 178/72 and LD842 was not due to undetected chromosomal mutations. Simon et al. (25) reported the use of a Tn*5* derivative containing the origin of transfer of the plasmid RP4 (closely related to RK2) in mobilizing large plasmids. This work also demonstrates the usefulness of the Tn*5-oriT* transposon for the genetic manipulation of pSDL2. Plasmids containing Tn*5-oriT* can be mobilized at a high frequency by an RK2 helper plasmid in a triparental mating system. This approach enabled us to transfer derivatives of pSDL2 into *S. dublin* to facilitate curing of the plasmid and to reintroduce the plasmid into the cured strain, thereby demonstrating its role in virulence.

The mechanism of virulence conferred by the *S. dublin* plasmid is not known. Jones et al. described a 60-megadalton plasmid in *S. typhimurium* that was associated with adhesion to and penetration into HeLa cells (16). This was thought to indicate an effect on invasiveness at the gut level. Our results indicate that the *S. dublin* plasmid exerts its effect at a different stage in pathogenesis. The finding of low but significant numbers of organisms in the spleens of mice infected orally with LD842 suggests that the plasmid-free strain can still invade the intestinal mucosa, but is then unable to establish a progressive systemic infection. This is confirmed by the lack of virulence in LD842 given by intraperitoneal inoculation, a model that bypasses the gut epithelium. pSD6 is able to restore virulence in both oral and intraperitoneal infections, indicating that the plasmid is required for *S. dublin* to establish a lethal systemic infection.

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