Physical and Genetic Mapping of the *Salmonella dublin* Virulence Plasmid pSDL2

Relationship to Plasmids from Other *Salmonella* Strains

Paul R. Beninger,* Gary Chikami,* Ken Tanabe,* Chantal Roudier,* Joshua Fierer,‡ and Donald G. Guiney*

*Department of Medicine, Division of Infectious Diseases H811F, University of California, San Diego Medical Center, San Diego, California 92103; and ‡Departments of Medicine and Pathology, Veterans Administration Medical Center, San Diego, California 92161

Abstract

Plasmids of ~ 80 kb in size are found in nearly all clinical isolates of *Salmonella dublin* and are believed to be essential for virulence. We have shown previously that the 80-kb plasmid pSDL2 is required for the *S. dublin* Lane strain to establish a lethal systemic infection in BALB/c mice after oral or intraperitoneal inoculation. We now present a physical and genetic characterization of pSDL2. We have established a complete restriction endonuclease cleavage map of pSDL2 for five enzymes: Xba I, Bam HI, Xho I, Sal I, and Hind III. The region specifying autonomous replication has been localized to a 10.5-kb region of the Sal I A fragment by subcloning on the vector pBR322. Using transposon insertion mutagenesis with Tn5-*ori*T, a region encoding the virulence phenotype has been mapped within a 6.4-kb portion of the Sal I B fragment. Deletions generated by partial Eco RI restriction digestion demonstrate that at least 50 kb of the plasmid DNA are not required for replication or virulence functions, confirming the map location of these phenotypes. Plasmids of different sizes and restriction patterns were found in mouse virulent strains of *S. dublin* Vi⁺, *S. enteritidis*, and *S. choleraesuis*. By Southern hybridization, these putative virulence plasmids share a common 4-kb Eco RI fragment with the virulence region of pSDL2, and the plasmids from *S. dublin* Vi⁺ and *S. enteritidis* were shown to express mouse virulence comparable to pSDL2.

Introduction

Plasmid-encoded gene products are required for the virulence of many pathogenic *Enterobacteriaceae* that cause enteric or systemic disease (1). Plasmid-mediated virulence traits have been firmly established for *Shigella* (2–4) and *Yersinia* (5–7) species, as well as toxigenic and enteroinvasive strains of *Escherichia coli* (8, 9). More recently, plasmids in the 30–60-MD size range have been implicated in the virulence of nontyphoid *Salmonella* serotypes, including *S. typhimurium* (10–12), *S. enteritidis* (13, 14), and *S. dublin* (11, 15, 16). In the case of *S. dublin*, a species that is host-adapted to cattle, nearly all clinical isolates contain plasmids of ~ 80 kb (55 MD) (11, 14–17), and a plasmid-free cured strain of *S. dublin* was shown by Terakado et al. (15) to have lost virulence for mice. Conclusive evidence for the role of plasmids in *S. dublin* virulence was provided by Chikami et al. (16), who labeled the plasmid from

a virulent *S. dublin* isolate with a derivative of the kanamycin transposon Tn5 and showed that reintroduction of the plasmid into both a cured strain and a naturally occurring plasmid-free isolate restored virulence for mice. Baird et al. (11) isolated two TnA transposon insertions that inactivated virulence in a similar *S. dublin* plasmid, and showed that this putative virulence region is homologous to an *S. typhimurium* plasmid. Current evidence suggests there is a family of virulence plasmids in *Salmonella* serotypes (17).

In both cattle and humans, *S. dublin* is particularly invasive, and oral infection frequently results in septicemia even without concomitant diarrhea (18–20). The course of infection that follows oral inoculation of *S. dublin* into BALB/c (*ity*ˢ) mice parallels the human infection, with colonization of the small bowel being followed by growth in Peyer's patches, mesenteric lymph nodes, and the spleen, ending in septicemia (16, 21). Heffernan et al. (21) compared the disease produced by the virulent, plasmid-containing *S. dublin* Lane strain with infection by a cured, plasmid-free derivative, designated LD842. Both Lane and LD842 colonized the bowel, multiplied in Peyer's patches, and spread to regional lymphatics and the spleen. However, only the Lane strain was able to cause progressive infection of the spleen and liver, while LD842 was contained by granuloma formation and eventually eliminated. Sublethal irradiation prevented the granulomatous inflammatory response and rendered the mice susceptible to infection with LD842, suggesting that mononuclear cells are essential for resistance to plasmid-free *S. dublin*. These in vivo results indicate that the virulence plasmid is not required for invasion of Peyer's patches nor for spreading to regional nodes and the spleen, but is necessary to establish a progressive infection of the reticuloendothelial system. Similar results have recently been reported for the virulence plasmids of *S. typhimurium* and a different isolate of *S. dublin* (22, 23).

This paper presents the physical and genetic characterization of the *S. dublin* virulence plasmid pSDL2. A restriction endonuclease cleavage map has been determined for five enzymes, and two important genetic functions have been mapped: the region specifying autonomous replication and the region encoding the virulence phenotype. Using a virulence region probe from pSDL2, the plasmids from *S. dublin*, *S. enteritidis*, and *S. choleraesuis* are shown to share a homologous 4-kb Eco RI fragment.

Methods

Growth media, bacterial strains, and plasmids. Trypticase soy broth (TSB)¹ (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville MD), L-broth (LB), Mueller-Hinton medium, and M9 medium served as growth media (24). *S. dublin* Lane (a nicotinamide

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1. *Abbreviations used in this paper:* LB, L-broth; *ori*T, origin of transfer; SSC, standard saline citrate; TSB, trypticase soy broth.

auxotroph) is a clinical isolate from a blood culture, identified by standard microbiologic criteria (25), and contains the 80-kb plasmid pSDL2 (16). LD842 is a derivative of *S. dublin* Lane from which pSDL2 has been cured (16). *S. dublin* 82/123, obtained from P. W. Jones, Compton, England, produces the Vi antigen and was identified and serogrouped by the California State Public Health Laboratory; the strain is auxotrophic for tryptophan. *S. dublin* 82/123 *nal*^{R} was a spontaneous nalidixic acid–resistant mutant selected for growth on 100 μg/ml of nalidixic acid. *S. enteritidis* 697 is a clinical isolate from a patient with gastroenteritis. *S. choleraesuis* 116 was obtained from R. W. Griffith (26). *E. coli* JA221 (*leu*B, Δ*trp*E5, *lac*Y, *rec*A, *hsd*R$^-$, *hsd*M$^+$) and *E. coli* C2110 *nal*^{R} (*pol*A, *his, rha*) have been previously described (27, 28). For triparental matings, the helper plasmid was maintained in *E. coli* MV12, a C600 Δ*trp*E5, *rec*A strain (29). The plasmid pBR322 Ap^r, Tc^r, a ColE1 type-replicon, has been well characterized (30). pEYDG1 is a pBR322 replicon, with deletion of its own origin of transfer$^-$ (*ori*T) and Tc^r gene, and carries a derivative of Tn5 which contains the *ori*T from the broad host range plasmid RK2 cloned into the Bam HI site of Tn5 without disruption of the kanamycin-resistance gene (31). The helper plasmid pRK2073 Tp^R Sm^R *tra*$^+$ is derived from pRK2013 by a Tn7 insertion in the kanamycin-resistance gene (31). pRK2013 was constructed by cloning the transfer region of RK2 together with kanamycin resistance into the Eco RI site of ColE1.

Restriction endonucleases. Xba I, Xho I, Bam HI, Sal I, Eco RI, and Hind III, and T4 DNA ligase were obtained from Bethesda Research Laboratories (BRL), Gaithersburg, MD. Reaction conditions conformed to the vendor's specifications.

Plasmid analysis. CsCl-purified plasmid DNA was prepared according to the method of Currier and Nester (32). Rapid isolation of plasmid DNA for clone analysis of *Salmonella* and *E. coli* strains was performed by a scaled-down modification of Currier and Nester's method as previously described (16).

Plasmid constructions and transformations. The replication region of pSDL2 was isolated by cloning in pBR322. Purified pSDL2 DNA (~ 0.5 μg) was completely digested with Sal I, mixed with 0.5 μg of Sal I–digested pBR322 DNA, and ligated in a volume of 20 μl. The ligation mix (1–2 μl) was transformed into *E. coli* C2110 using the standard CaCl$_2$ technique originally described by Cohen et al. (33). The cells were plated on LB agar containing penicillin (200 μg/ml) and individual colonies were repicked onto penicillin plates, grown overnight in 5 ml of LB that contained penicillin, and analyzed for plasmid DNA (16). To isolate Eco RI deletions of pKT2 and pKT4, the plasmids were purified by CsCl/ethidium bromide density gradient centrifugation. Approximately 0.5 μg of plasmid DNA was digested with Eco RI, then ligated in a volume of 100 μl and transformed into C2110 selecting for penicillin resistance as described above. Eco RI deletions of the pSDL2::Tn5-*ori*T plasmids 9–18 and 16–11 were constructed similarly, except that partial Eco RI digestions were used for the ligations.

Isolation of Tn5-oriT insertions in Salmonella plasmids. The genetic procedures for isolating and characterizing Tn5-*ori*T transpositions into pSDL2 were modified from our previously published techniques (16). The manipulations are divided into five steps. (*a*) pEYDG1 (carrying the Tn5-*ori*T transposon) was transferred into *S. dublin* Lane containing pSDL2 by conjugation using the helper plasmid pRK2073. Strain *E. coli* JA221 (pEYDG1) was grown in LB with kanamycin (50 μg/ml), strain *E. coli* MV12 (pRK2073) was grown in Mueller-Hinton broth with trimethoprim (100 μg/ml), and *S. dublin* Lane was grown in LB. Strains were harvested in late log phase, washed once in normal saline, resuspended in normal saline to the original volume, and 1-ml vol of each were mixed together and passed through a 0.45-μm filter (Millipore Continental Water Systems, Bedford, MA). The filter was incubated cell-side up on an LB agar plate at 37°C for 1 h. The bacteria were washed off the filter by vortexing in normal saline, and serial 10-fold dilutions were plated on M9-medium containing kanamycin (50 μg/ml) to select for *S. dublin* Lane containing pEYDG1. 100 colonies from these plates were repicked and tested for trimethoprim resistance (pRK2073); sensitive colonies were saved for

the next step. (*b*) Transpositions of Tn5-*ori*T from pEYDG1 to pSDL2 were selected by a second conjugal transfer from *S. dublin* into *E. coli* C2110 (*pol*A) *nal*^{R} in which pSDL2::Tn5-*ori*T plasmids can replicate, but pEYDG1 and pRK2073 cannot replicate. A colony of *S. dublin* Lane containing both pSDL2 and pEYDG1 from step *a* was grown to late log phase in LB containing kanamycin, washed, and mixed with *E. coli* MV12 (pRK2073) and *E. coli* C2110 *nal*^{R} according to the mating procedure described above. After mating, the cells were plated on LB agar that contained 50 μg/ml kanamycin and 25 μg/ml nalidixic acid to select for C2110 *nal*^{R} colonies containing pSDL2::Tn5-*ori*T plasmids. (*c*) The pSDL2::Tn5-*ori*T plasmids were analyzed in C2110 by the rapid clone analysis procedure. Plasmid DNA was digested with Sal I and analyzed by agarose gel electrophoresis to locate the Tn5-*ori*T insertions within individual Sal I fragments. The precise location of the Tn5-*ori*T inserts in the Sal I B fragment was determined from Bam HI and Hind III digestions. (*d*) Individual pSDL2::Tn5-*ori*T plasmids designated for virulence testing were transformed into JA221, a *trp*$^-$ *E. coli* strain. (*e*) The pSDL2::Tn5-*ori*T plasmids in JA221 were transferred by conjugation into the plasmid-free, avirulent *S. dublin* LD842 strain, using the helper plasmid pRK2073. Midlog phase cells of JA221 (pSDL2::Tn5-*ori*T), MV12 (pRK2073), and *S. dublin* LD842 were harvested, washed, and mixed together as described above. After mating, LD842 colonies containing pSDL2::Tn5-*ori*T plasmids were selected on M9 agar with 100 μg/ml nicotinamide and 50 μg/ml kanamycin. These colonies were screened for trimethoprim resistance (pRK2073), and sensitive clones were saved for stability testing. A similar procedure was used to isolate and transfer Tn5-*ori*T insertions in pSDV1 and pSE1. Since *S. dublin* 82/123 Vi$^+$ is a tryptophan auxotroph, the scheme was modified as follows: pEYDG1 was transferred into *S. dublin* 82/123 Vi$^+$ *nal*^{R} using nalidixic acid (20 μg/ml) to counterselect against the *E. coli* donors. pSDV1::Tn5-*ori*T plasmids were then isolated by mating into *E. coli* C2110 with plating on M9 agar containing 50 μg/ml kanamycin.

In vitro stability testing. Each *S. dublin* LD842 strain containing a plasmid insertion mutant was tested for stability of the plasmid construct. The strain was grown in LB at 37°C without antibiotic selection for ~ 30 generations. The cells were diluted and replica plated on LB-agar and LB-agar + kanamycin. Stable mutants demonstrated < 2% loss. Unstable mutants demonstrated > 50% loss. About one-half of the plasmids with Tn5-*ori*T inserts were unstable and could not be used for virulence testing.

Mouse infection. To test the virulence of *S. dublin* LD842 containing plasmid transposon insertions, strains were grown overnight with kanamycin selection (50 μg/ml) in TSB at 37°C, washed in normal saline, and resuspended in TSB containing kanamycin to an optical density that corresponded to 10^9 CFU/ml. Each was further diluted in normal saline to 10^4 CFU/ml just before inoculation, and 0.1 ml was injected intraperitoneally, which represented 10^2 × LD$_{50}$ for *S. dublin* Lane (16). There were three or five female BALB/c (*ity*^{s}) mice (15–18 g) per group and groups were caged separately.

There was always a positive control group of mice injected intraperitoneally with *S. dublin* LD842 containing pSD6, a virulent Tn5-*ori*T insertion derivative of pSDL2 (16). Mice were allowed access to food and water and observed for 14 d. All positive control mice died between day 5 and day 8. All survivors after 14 d were killed and their spleens were cultured in duplicate on TS agar and TS agar + kanamycin. Colony counts were within 10% for each pair of plates, and retention of the transposon-labeled plasmids was confirmed by the rapid plasmid analysis procedure.

DNA hybridization. The standard procedures for agarose gel electrophoresis and transfer of DNA fragments to nitrocellulose were performed as described previously (34). The virulence region probe consisted of the 4-kb Eco RI fragment contained within the Sal I B fragment of pSDL2 (see text and Fig. 4). The probe fragment was purified by agarose gel electrophoresis from an Eco RI digest of pSD15.2, which was constructed by ligating the Sal I B fragment of pSDL2 into the Sal I site of pBR322. The probe was labeled with biotin-7-dATP using nick-translation reagents from BRL. Hybridization, washes, and color

development reactions were performed according to a protocol supplied by BRL (BluGene system). Hybridization was done overnight using 45% formamide (vol/vol) at 42°C, with successive washes in 2× standard saline citrate (SSC), 0.1% (wt/vol) SDS at room temperature; 0.2× SSC, 0.1% SDS at room temperature; and 0.16× SSC, 0.1% SDS at 50°C.

Results

Endonuclease restriction map. We screened 18 different restriction enzymes for the number of sites present on pSDL2, and selected five enzymes (Xba I, Xho I, Bam HI, Sal I, and Hind III) for detailed mapping studies. Agarose gel electrophoretic patterns of pSDL2 digested with Xba I, Xho I, Bam HI, and Sal I are shown in Fig. 1. The restriction pattern of pSDL2 with Hind III has been published (16). There is a single Xba I site, three Xho I sites, three Bam HI sites, and seven Sal I sites (the third band of the Sal I digest is a doublet). Digestion with Hind III (not shown) results in nine sites (16). The Xba I site provides the reference point for constructing the restriction map shown in Fig. 2. A single digest of pSDL2 with Xho I was run in parallel with a double digest of pSDL2 with Xba I and Xho I. The 15.4-kb Xho I fragment is cleaved by Xba I into 12.0- and 3.4-kb subfragments. Similar parallel single and double digests oriented Xba I with respect to Bam HI, Sal I, and Hind III. A timed, partial digest of pSDL2 with Sal I identified the 6.5-kb E and F partial digest product, which showed that E and F are contiguous. The order of the Sal I B, D, E, and F fragments was determined from digestion of a large 36-kb Bgl II fragment which spans this region (not shown).

In the next step, the Sal I fragments were individually isolated on agarose gels. The Sal I A fragment, 28.5 kb, contains a single Eco RI site, yielding subfragments of 18 and 10.5 kb (shown in Figs. 3 and 4). Restriction with Hind III resulted in
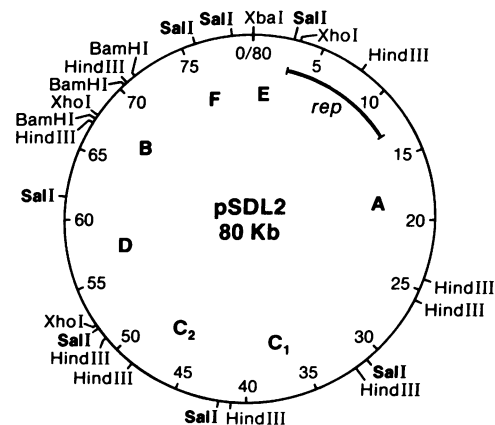

Figure 2. Endonuclease restriction map of pSDL2. The size is 80 kb, which was derived from adding the sizes of individual fragments. It is oriented with respect to Xba I, a unique restriction site. Restriction sites are also shown for the three Xho I sites, three Bam HI sites, seven Sal I sites, and nine Hind III sites. *rep* represents the region associated with replication.

four subfragments: 16.2, 5.7, 5.2, and 1.4 kb. Comparison with the Hind III digest of pSDL2 and the large Eco RI-Sal I portion of the A fragment oriented the Hind III sites as shown in Figs. 2 and 4.

A similar strategy was employed with the Sal I B fragment. The distances from Xba I to the Bam HI, Hind III, and Xho I sites were determined by double digestions. The Sal I B fragment was gel purified and digested with Bam HI, Xho I, and Hind III. The internal Hind III G fragment was identified, as well as both internal Bam HI fragments. These two small Bam HI fragments (3.2 and 0.8 kb) were purified and digested
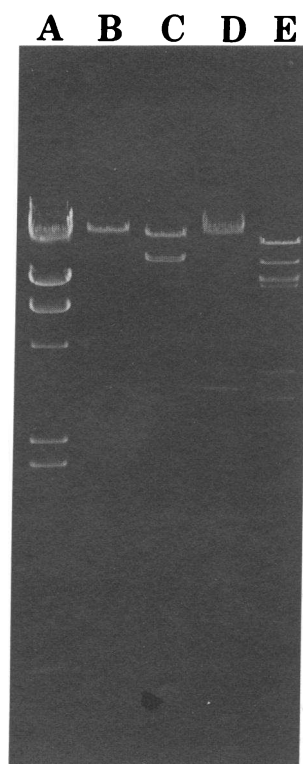

Figure 1. Agarose gel (0.7%) electrophoretic patterns of the restriction endonuclease fragments of pSDL2. Lane *A*, a Hind III digest of lambda, serves as a size reference standard. Lanes *B, C, D,* and *E* are digests of pSDL2 with Xba I, Xho I, Bam HI, and Sal I, respectively. The third largest Sal I band is a doublet.
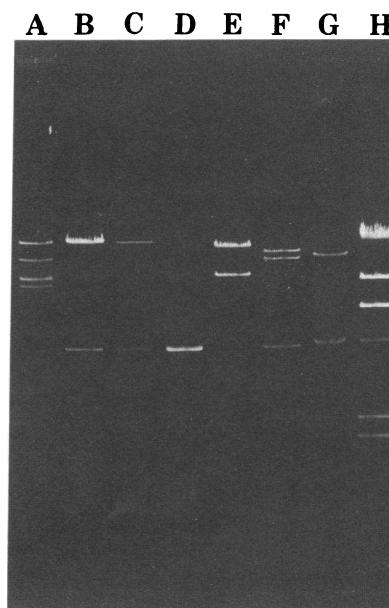

Figure 3. Agarose gel (0.7%) electrophoretic patterns of restriction fragments from pKT2, pKT4, and pKT4Δ202. Lane *A*, Sal I digest of pSDL2, showing the Sal I A, B, $C_1 + C_2$, D, E, and F fragments in decreasing size; lane *B,* Sal I digest of pKT2; lane *C,* Sal I digest of pKT4; lane *D,* Sal I digest of pBR322; lane *E,* Eco RI digest of pKT2 showing the two fragments depicted in Fig. 4; lane *F,* Eco RI digest of pKT4 showing the presence of a unit length copy of pBR322 as well as the two Eco RI fragments derived from the Sal I A cloned fragment; lane *G,* Eco RI digest of pKT4Δ202 showing loss of the large Eco RI fragment; and lane *H,* Hind III digest of lambda DNA.

A

Sal I   Hind III   EcoRI   Hind III Hind III   Sal I
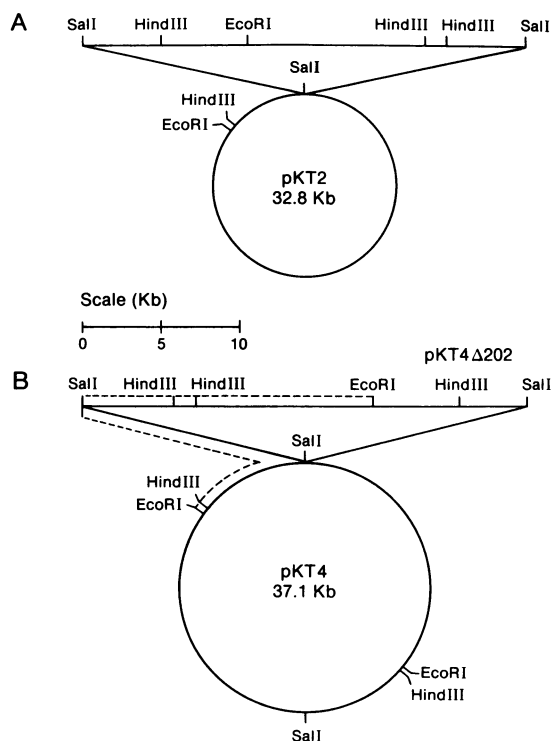
Figure 4. Endonuclease restriction maps of pKT2, pKT4, and pKT4Δ202. These plasmids were selected for the ability to replicate in *E. coli* C2110, as described in the text. (*A*) pKT2 is a 32.8-kb plasmid containing the entire pSDL2 Sal I A fragment cloned into the unique Sal I site of pBR322. (*B*) pKT4 is a 37.1-kb construct of pSDL2 Sal I A, with the opposite orientation to pKT2, cloned into a Sal I site of a dimer of pBR322. pKT4Δ202 represents an Eco RI deletion clone, which further localized the region of replication to a Sal I-Eco RI 10.5-kb fragment. The dashed line indicates the DNA deleted.

with Xho I and Hind III to localize these sites. Using double digestions of purified fragments, the additional Xho I sites were shown to be just inside the Sal I A and Sal I D fragments. Since the order of the Sal I A, B, D, E, and F fragments is known, then Sal I C_1 and C_2 must lie next to each other between the A and D fragments. The Hind III sites in this region were determined by fragment purification and double digestions.

Isolation of the replication region of pSDL2. The region of pSDL2 that encodes autonomous replication and maintenance properties was isolated by subcloning on pBR322, a ColEl-type plasmid which is unable to replicate in the DNA polymerase I–deficient (*pol*A) strain *E. coli* C2110. Insertion of the region containing the replication system of pSDL2 into pBR322 allows the hybrid plasmid to replicate and express penicillin resistance in C2110. Accordingly, a complete Sal I digest of pSDL2 was ligated to Sal I–digested pBR322 and the mixture was transformed into C2110 selecting for penicillin resistance. Plasmids isolated from 24 separate resistant clones were digested with Sal I and examined by agarose gel electrophoresis. Most plasmids contained several Sal I fragments, but all plasmids had in common the Sal I A fragment in addition to pBR322. Two constructs containing only the Sal I A fragment and pBR322 were selected for further study and designated pKT2 and pKT4 (Figs. 3 and 4). Hind III and Eco RI restrictions of pKT2 and pKT4 demonstrated that the pSDL2

Sal I A fragment is in both of the two possible orientations with respect to pBR322, and that pKT4 contains an additional copy of pBR322. Using the unique Eco RI restriction site on pSDL2 Sal I A and the unique Eco RI site on pBR322, deletion clones were constructed. Using the same strategy described above, a complete Eco RI digest of either pKT2 or pKT4 was religated to itself, then the mixture was used to transform C2110, and the transformants were selected for penicillin resistance. None of the pKT2 penicillin-resistant transformants evaluated by clone analysis and Eco RI restriction had lost the smaller 10.5-kb Eco RI-Sal I portion of the Sal I A fragment from pKT2. However, transformants using pKT4 contained deletions of the larger 18-kb Eco RI-Sal I portion of the Sal I A fragment (Fig. 3). This deletion derivative, designated pKT4Δ202 (Fig. 4), demonstrates that the remaining 10.5 kb of the Sal I A fragment contains the region of pSDL2 necessary for replication and maintenance of the plasmid.

Location of the virulence region of pSDL2. We used transposon insertion mutagenesis to locate and map the virulence region on pSDL2. The Tn5-*ori*T transposon, a derivative of Tn5 containing the transfer origin of the conjugative plasmid RK2, is particularly useful because it allows mutated pSDL2 derivatives to be transferred by mating into *S. dublin* for virulence testing. Tn5-*ori*T insertions into pSDL2 were isolated as described in Methods. Selected mutants were transferred into the plasmid-free, avirulent *S. dublin* LD842 strain. 28 unique, stable Tn5-*ori*T mutants of pSDL2 were tested for virulence in mice by intraperitoneal inoculation of 10^3 organisms. Two groups of mutant strains were clearly identified by these experiments: the virulent strains killed all the mice within 8 d while the avirulent strains killed none of the mice within 14 d. Each avirulent strain was reisolated from the spleens of mice killed after 14 d and was shown to contain the pSDL2::Tn5-*ori*T plasmid. This control showed that avirulence was due to the transposon insertion and not to loss of the plasmid in vivo. Mutants were initially selected for inserts that represented an even distribution about pSDL2, but once we had identified an avirulent mutant with its Tn5-*ori*T insert in the Sal I B fragment, we undertook an intensive effort to define the limits of that virulence region by selecting mutants with inserts concentrated in the Sal I B fragment. Fig. 5 represents a detailed map of the pSDL2 Sal I B fragment showing the Tn5-*ori*T inserts that were tested for virulence. Five mutants are avirulent. Their respective inserts are pSD15-1, 16-8, 13-4, 14-1, and 15-2, which set an inner limit of 4.2 kb for the region encoding virulence. Two virulent inserts, pSD13-3 and 9-18, which are adjacent to the avirulent mutants pSD15-1 and 15-2 in the Sal I B fragment, set an outer limit of 6.4 kb for the virulence region. All of the other 23 mutants are virulent. The number of virulent inserts in the Sal I fragments are distributed as follows: Sal I A:5, Sal I B:5, Sal I C_{1+2}:8, Sal I D:3, Sal I E:0, and Sal I F:2.

Eco RI deletions of pSDL2 that replicate and express virulence. To confirm the location of the replication and virulence regions of pSDL2, we isolated a series of deletion derivatives generated by partial Eco RI digestion. We used two derivatives of pSDL2 that contained Tn5-*ori*T insertions adjacent to the virulence region: pSD9-18 and pSD16-11 (Fig. 5). These plasmids were partially digested with Eco RI, ligated, and transformed separately into *E. coli* JA221 selecting for kanamycin resistance. Several clones containing deletions were mapped and three are shown in Fig. 6. These derivatives were
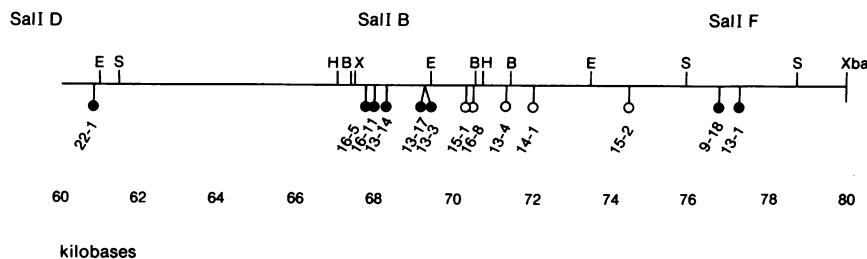
Sall D   Sall B   Sall F

60   62   64   66   68   70   72   74   76   78   80

kilobases

Figure 5. Map of Tn5-*ori*T insertion mutants in the region required for virulence. ●, location of inserts that failed to abolish virulence (see text for Methods); ○, inserts that abolished virulence expression. *E*, Eco RI; *H, Hind* III; *X*, Xho I; *B*, Bam HI; and *S*, Sal I. The Eco RI sites were localized in this region by gel purification of the Sal I B fragment and selected subfragments followed by digestion with Eco RI.

transferred by conjugation into *S. dublin* LD842 and tested for virulence in mice as described in Methods. pSD9-18Δ11 could not be maintained in LD842; all the kanamycin-resistant transconjugants that were analyzed contained Tn5-*ori*T in the chromosome. The two other constructs were stable in LD842 and restored the mouse virulence of this strain. Both pSD9-18Δ7 and pSD16-11Δ2 lost ~ 50 kb of DNA but retained stable replication and virulence properties as predicted from the cloning and Tn5-*ori*T insertion data in Figs. 4 and 5.

Homology between the virulence region of pSDL2 and plasmids in different strains of Salmonella. We had previously examined 16 different isolates of *S. dublin* and found plasmids similar to pSDL2 by size and restriction pattern in each strain (16). We subsequently analyzed an *S. dublin* isolate, designated 82/123, that produced Vi antigen and had lower virulence in mice (LD$_{50}$ of 10^3 by intraperitoneal injection) than *S. dublin* Lane. This strain contained a single plasmid of 60 kb (pSDV1) with a restriction pattern considerably different from pSDL2. In addition, we analyzed the mouse virulent strains *S. enteritidis* 696 and *S. choleraesuis* 116 for the presence of putative virulence plasmids. *S. enteritidis* 696 contained a single 60-kb plasmid (pSE1) while *S. choleraesuis* had two plasmids of 36 and 8 kb (designated pSCH1 and pSCH2). Eco RI restriction digests of these plasmids together with pSDL2 are shown in Fig. 7 *A;* pSDL2, pSDV1, pSE1, and pSCH1 differ substantially in restriction pattern, and pSCH2 contains a single Eco RI site. To examine the homology of these plasmids with the virulence region of pSDL2, the 4-kb Eco RI fragment defined by the avirulent Tn5-*ori*T inserts 15–1, 16–8, 13–4, and 14–1 (Fig. 5) was labeled and used as a hybridization probe with the Southern blot of the gel shown in Fig. 7 *A;* the

result, shown in Fig. 7 *B*, demonstrates that pSDV1, pSE1, and pSCH1 all contain 4-kb Eco RI fragments with strong homology to the virulence region of pSDL2. The small *S. choleraesuis* plasmid pSCH2 lacks homology.

A similar hybridization study was done using as a probe the 10.5-kb Eco RI-Sal I fragment from pKT4Δ202, and encoding the replication region of pSDL2 (Fig. 4). Homologous sequences were found in each of the other *Salmonella* virulence plasmids tested: pSDV1, pSE1, and pSCH1, although in these plasmids the homologous Sal I fragment was 7.5 kb as opposed to 28 kb for the Sal I A fragment of pSDL2 (data not shown).

Virulence of pSDV1 and pSE1 in S. dublin LD842. Since pSDV1 and pSE1 contain sequences homologous to the virulence region of pSDL2, we studied the virulence conferred by these plasmids in LD842. We isolated Tn5-*ori*T inserts in both pSDV1 (pSDV213) and pSE1 (pSE707), then transferred the derivatives separately into LD842 by conjugation. As shown in Table I, both pSDV213 and pSE707 render LD842 as virulent as the wild-type *S. dublin* Lane that contains pSDL2.

Discussion

The restriction endonuclease map of pSDL2 presented in Fig. 2 permits localization and genetic mapping of two important plasmid functions and provides the basis for comparison with other *Salmonella* plasmids. The map agrees well with the partial restriction map of sites in and around the Sal I B fragment of the *S. dublin* plasmid 2229, which was reported by Baird et al. (11), indicating close homology between these independent isolates from different continents. The Hind III restriction fragments of pSDL2 are similar or identical to the patterns of
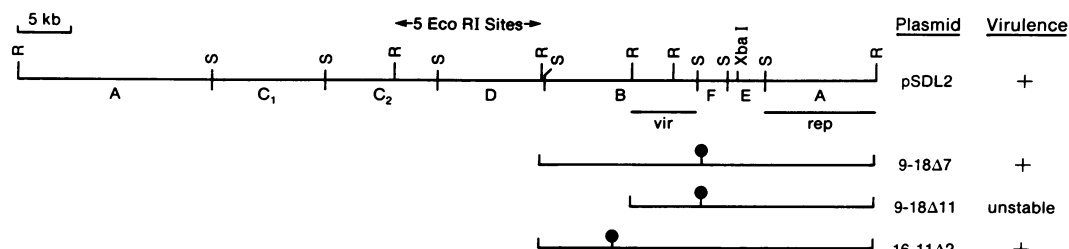

Figure 6. Map of Eco RI-generated deletions of pSDL2. *R*, Eco RI sites; *S*, Sal I sites. The Sal I fragments are labeled *A* to *F* to correspond to the map in Fig. 2. The exact map location of a cluster of Eco RI sites in the C$_2$ and D fragments has not been determined, but all of these sites have been lost in the deletion derivatives shown here. The sites of transposon insertions 9–18 and 16–11 (from Fig. 5) are shown as closed circles. Virulence was determined as described in Methods.

Plasmid	Virulence
pSDL2	+
9-18Δ7	+
9-18Δ11	unstable
16-11Δ2	+
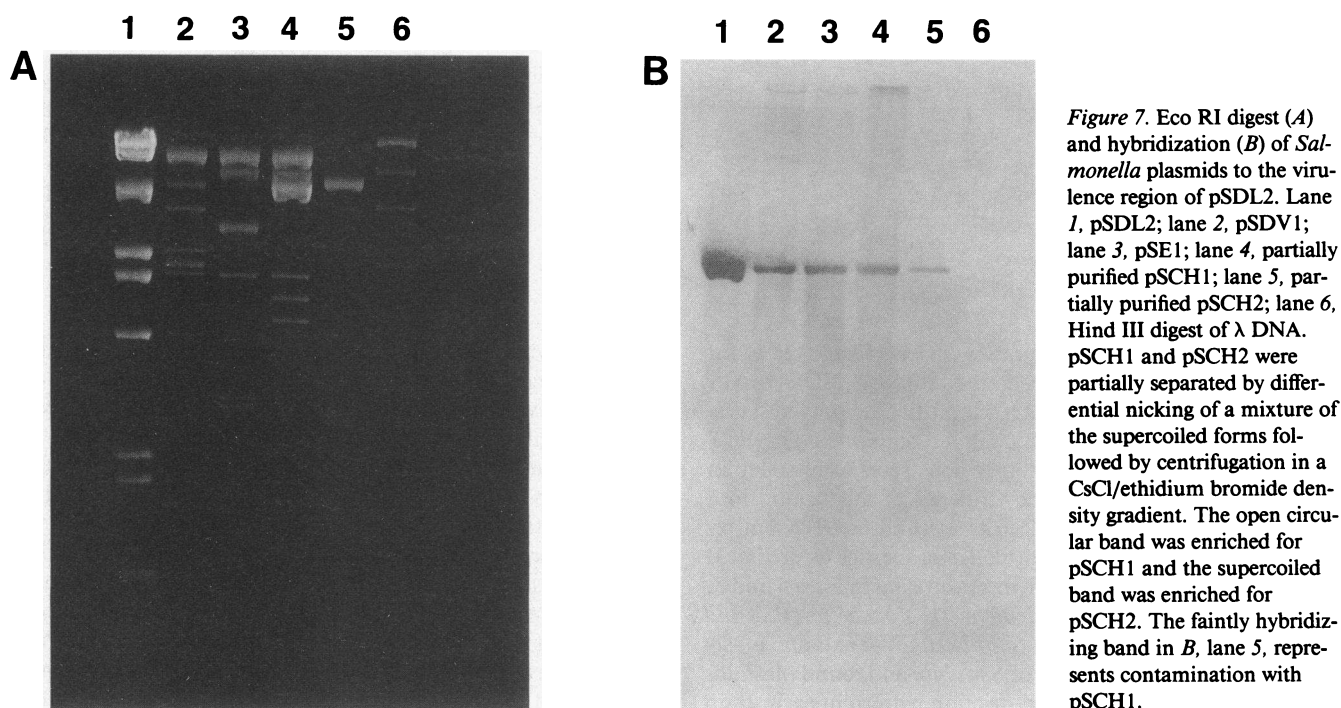
Figure 7. Eco RI digest (A) and hybridization (B) of Salmonella plasmids to the virulence region of pSDL2. Lane 1, pSDL2; lane 2, pSDV1; lane 3, pSE1; lane 4, partially purified pSCH1; lane 5, partially purified pSCH2; lane 6, Hind III digest of λ DNA. pSCH1 and pSCH2 were partially separated by differential nicking of a mixture of the supercoiled forms followed by centrifugation in a CsCl/ethidium bromide density gradient. The open circular band was enriched for pSCH1 and the supercoiled band was enriched for pSCH2. The faintly hybridizing band in B, lane 5, represents contamination with pSCH1.

the Japanese isolates reported by Terakado et al. (15) and the French isolates of Popoff et al. (17), but differ from the S. dublin plasmids studied by Helmuth et al. (14). In addition to containing atypical plasmids, the S. dublin strains reported by Helmuth et al. have a different phenotype compared with strains isolated in California (25). Strains studied by Helmuth et al. are not auxotrophic, ferment arabinose, and are not lysed by phage ES_{18} (35; Fierer, J., unpublished results). Thus, there appears to be considerable conservation of sequences among certain S. dublin plasmids from different parts of the world, but more than one type of plasmid may exist.

The replication region of pSDL2 is located in the 10.5-kb Sal I-Eco RI fragment shown in Figs. 2 and 4. Replication is a key function that determines the incompatibility, host range, and stable maintenance of the plasmid in different bacteria. pSDL2 is exceedingly stable in S. dublin (16). The replication fragment of pSDL2 hybridizes to regions in each of the other Salmonella virulence plasmids pSDV1, pSE1, and pSCH1,

Table I. Virulence of pSDV1 and pSE1 in S. dublin LD842

Strain	Dose	Deaths/total*
pSDV1 virulence		
LD842 (pSDV213)	2.4×10	3/3
	2.4×10^2	3/3
S. dublin Lane (pSDL2)	2.7×10	3/3
	2.7×10^2	3/3
pSE1 virulence		
LD842 (pSE707)	3×10	4/5
	3×10^2	5/5
S. dublin Lane (pSDL2)	3×10	3/5
	3×10^2	5/5

* Mice were inoculated intraperitoneally and followed for 14 d as described in Methods.

which suggests that these plasmids contain a common replication and maintenance system.

A region required for the virulence phenotype of pSDL2 is defined by the Tn5-oriT inserts shown in Fig. 5. The location of both the replication and virulence regions was confirmed by the isolation of the Eco RI deletions shown in Fig. 6. The virulence of plasmids 9–18Δ7 and 16–11Δ2 demonstrates that ~ 50 kb of pSDL2 is not required for expression of the virulence phenotype. A similar result has recently been reported for the 90-kb plasmid of S. typhimurium: virulence and primary replication functions map together in a 40-kb region (36). Baird et al. (11) mapped two TnA transposon insertions that decrease virulence in their S. dublin strain 2229 plasmid. One insert is at about 72 kb on the map in Fig. 5, very close to the avirulent Tn5-oriT insert 14–1. The other avirulent TnA insertion was at about 66 kb, within the Eco RI fragment that was deleted in pSD9–18Δ11 (Fig. 6). This TnA insertion could have affected the stability of their plasmid, since our results indicate that loss of this Eco RI fragment makes pSD9–18Δ11 very unstable in S. dublin.

The virulence region defined by the Tn5-oriT inserts is highly conserved among other Salmonella plasmids, as seen in Fig. 7. Despite differences in size and the pattern of restriction enzyme fragments, all of the plasmids studied contain a highly homologous 4-kb Eco RI fragment. This result suggested that these regions encoded similar virulence functions in the different plasmids. This hypothesis was confirmed when we were able to restore the virulence of LD842 by transfer of Tn5-oriT derivatives of pSDV1 and pSE1.

Strain 82/123 is an unusual dublin isolate in that it produces Vi antigen, is a tryptophan auxotroph, and contains the pSDV1 plasmid with a considerably different restriction pattern compared with pSDL2 and 16 other S. dublin plasmids previously examined (16). However, the homology and mouse inoculation studies clearly show that pSDV1 encodes normal virulence properties in LD842. Furthermore, the ability to

confer virulence on LD842 is not limited to plasmids of serotype *dublin* strains, since pSE1 from *S. enteritiditis* also renders LD842 virulent. These results suggest that despite differences in size and restriction enzyme digest patterns of plasmids isolated from different *Salmonella* serotypes, all these plasmids encode virulence functions by a common genetic mechanism.

Acknowledgments

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