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Tn*1725* Transposon Mutagenesis of 9-18Δ7, an *Eco*RI Deletion Derivative of *Salmonella dublin* Lane Plasmid pSDL2

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A 37.5-kb derivative of the *Salmonella dublin* virulence plasmid pSDL2 was subjected to mutagenesis with the transposon Tn*1725*. Fifty-two insertions were mapped, and the mutants were tested for their ability to restore virulence to a plasmid-free strain of *S. dublin*. Twenty-nine of these inserts could not restore full virulence and thus define nine regions of the plasmid essential for virulence. Deletion of a 4.5-kb region by *Bal*31 nuclease resulted in a 33-kb derivative that maintained full virulence.

Though primarily a pathogen of cattle, *Salmonella dublin* is an infrequent cause of disease in humans. Patients harboring *S. dublin* often present with bacteremia and metastatic infection (7). *S. dublin*, *Salmonella cholerasuis*, and *Salmonella typhimurium* are pathogenic in a murine model, and their plasmids share a homologous 4-kb *Eco*RI fragment that is essential for virulence (19). The mechanism of pathogenesis of these bacteria is poorly understood.

The 80-kb plasmid pSDL2 of *S. dublin* Lane is essential for the expression of virulence in BALB/c mice. pSDL2 was originally labelled with the transposon Tn*5-oriT* to allow its transfer into *Escherichia coli* (4). To locate the regions necessary for virulence, pSDL2::Tn*5-oriT* plasmids were screened for their ability to restore virulence to the plasmid-free derivative of *S. dublin* Lane, LD842. Five inserts which inactivated virulence were found and were located in the 14.5-kb *Sal*I B fragment (see Fig. 1). However, the *Sal*I B fragment alone was unable to restore virulence. To determine whether larger segments of DNA flanking the region defined by the Tn*5-oriT* inserts were required for the expression of virulence, a series of deletion derivatives of pSDL2 were constructed. 9-18Δ7 and 16-11Δ2 were the smallest plasmids which restored virulence to LD842. They are 37.5 kb in size and consist of three *Eco*RI fragments contiguous in pSDL2 (2). In the present study, we have extended these observations by mapping regions of the 9-18Δ7 plasmid which are necessary for virulence.

Restriction endonucleases were obtained from Bethesda Research Laboratories, Gaithersburg, Md. Reaction conditions were as specified by the vendor. *Bal*31 digestions were performed under appropriate reaction conditions at 30°C for various periods of time, depending upon the extent of digestion required. The enzyme was inactivated by the addition of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and then phenol extracted. Blunt ends were created by treating the fragments with Klenow fragment (Bethesda Research Laboratories), and the fragments were recircularized by ligation (1). For *Bal*31 digests, 16-11Δ2 instead of 9-18Δ7 was used. 16-11Δ2 differs from 9-18Δ7 only in having the Tn*5* insert located further from the unique *Xba*I site (see Fig. 1), allowing digests to proceed without loss of Tn*5* and antibiotic selection.

Plasmid DNA was purified by the method of Currier and Nester (5). Rapid isolation of plasmid DNA for clone analysis of *S. dublin* was done by a scaled-down modification of the Currier and Nester procedure (4). Plasmid isolation for clone analysis of *E. coli* was done by the cleared lysate procedure (20).

Transformation of *E. coli* RU4404, which is *E. coli* MM294 (Cm^r *thi endA hsdR*) with Tn*1725* in the chromosome (21), with 9-18Δ7 was done by the method of Hanahan (9) with the following modification. After the competent cells were incubated with the transforming DNA, 0.8 ml of SOC (SOB with 1% glucose [9]) with kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml) was added and the transformants were incubated at 37°C with shaking for 1 h. Luria-Bertani broth (LB) (5 ml) with kanamycin and chloramphenicol was added, and the transformants were incubated at 30°C for 48 to 72 h to allow transposition. Transposition of Tn*1725* is optimum at 30°C because of temperature-sensitive transposase (20). Plasmids containing Tn*5-oriT* were mobilized by cotransfer with a helper plasmid in *E. coli* MV12 (C600 Δ*trpEC recA*), pRK2073, a ColE1 replicon expressing TMP^r which contains the RK2 transfer system (12). The donor, helper, and recipient strains (*E. coli* C2110 Nal^r *polA* [4]) were grown to late log phase in LB broth. The cells were then washed once in normal saline and resuspended in normal saline to their original volume. One-milliliter volumes of each were combined and collected on a filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The filter was incubated overnight on LB agar without selection, and then a sample was streaked out on media with chloramphenicol, kanamycin, and nalidixic acid (20 μg/ml). The target plasmids which received a copy of Tn*1725* were mated out of *E. coli* RU4404 into *E. coli* C2110 Nal^r. Plasmid DNA with Tn*1725* inserts of interest was transformed into *E. coli* JA221 (*leuB* Δ*trpE5 lacY recA hsdR hsdM*$^+$) (4) and then mated into *S. dublin* LD842 (*S. dublin* Lane cured of pSDL2 [4]) by using the helper, pRK2073, with selection on M9 plates containing 0.5% Casamino Acids, 0.2% glucose, 0.1 μg of nicotinamide per ml (16), chloramphenicol, and kanamycin. Following serial passage on M9 plates, loss of the helper plasmid was verified by loss of TMP^r.

Each *S. dublin* strain containing a plasmid Tn*1725* insert of

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interest was tested for the in vitro stability of the plasmid construct. The plasmid strain was grown in LB broth at 37°C without antibiotic selection for approximately 30 generations. The cells were diluted and replica plated on LB agar without antibiotics and LB agar with chloramphenicol and kanamycin. By this assay all reported mutants were stable and demonstrated less than 2% loss of the plasmid.

The virulence of these *S. dublin* strains was tested by lethality to BALB/c mice. *S. dublin* with 9-18Δ7::Tn*1725* was grown overnight in LB broth with chloramphenicol and kanamycin. Bacteria were recovered by centrifugation and washed once in normal saline. The concentration of organisms was adjusted to 10^4 CFU/ml in saline, and 0.1 ml was injected intraperitoneally into female BALB/c mice weighing 16 to 20 g each. Serial 10-fold dilutions and colony counts were performed to confirm the dose of bacteria given. Three animals were used per group, and the animals were observed for 2 weeks. *S. dublin* LD842(9-18Δ7) and *S. dublin* LD842 were used as positive and negative controls, respectively. This dose represents 100 times the 50% lethal dose (LD_{50}) for *S. dublin* Lane or LD842(9-18Δ7) (2). At this dose, mice infected with *S. dublin* LD842(9-18Δ7) begin to die at day 5 and all are dead by day 8. Mice injected with LD842 show no ill effects. Mice surviving the challenge with *S. dublin* were sacrificed at day 14. *S. dublin* LD842(9-18Δ7::Tn*1725*) bacteria, labelled 1 through 8 in Fig. 1, were further tested for attenuation by intraperitoneal injection of 5×10^4 and 1×10^6 bacteria. For this experiment, positive controls were injected with 5×10^4 *S. dublin* LD842(9-18Δ7) cells while negative controls were injected with 1×10^6 *S. dublin* LD842 cells. LD_{50}s were determined by intraperitoneal infection for the *Bal*31 digests. Six animals were tested at each of six dose levels from 10^1 to 10^6 bacteria. Positive controls were injected with 10 *S. dublin* LD842(9-18Δ7) cells, and negative controls were injected with 10^6 *S. dublin* LD842 cells. The LD_{50} was calculated by the method of Miller and Tainter (17).

The target replicon 9-18Δ7 carries a copy of Tn*5-oriT*. Tn*1725* carries Cm^r and is a member of the Tn*3* family of transposons (11). It is unrelated to Tn*5*, and therefore its insertion into 9-18Δ7 is not affected by the copy of Tn*5-oriT* already present. The transposition frequency is about 10^{-3} per cell, and the frequency of precise or imprecise excision ranges from 10^{-9} to 10^{-10} per cell (21). Since *E. coli* RU4404 is Rec^+, transposons can participate in a number of homologous recombination events. Of 612 clones of *E. coli* C2110 produced by the methods described above, transposition occurred as an uncomplicated event in 601. Of this number, 52 independent Tn*1725* inserts into 9-18Δ7, excluding inserts into Tn*5*, could be distinguished from possible siblings by mapping. Figure 1 shows a genetic map of 9-18Δ7. The open circles define the Tn*1725* inserts which were shown to inactivate virulence (none of three mice injected with 10^3 bacteria died), and the solid circles define inserts which had no effect on virulence (three of three mice died). For all 52 9-18Δ7::Tn*1725* plasmids, *S. dublin* was recovered from the spleen, and each plasmid was tested by restriction enzyme analysis for identity to the one originally mapped in *E. coli* C2110. All mice injected with 5×10^4 *S. dublin* LD842(9-18Δ7::Tn*1725*, numbers 1 through 7 in Fig. 1) cells survived. One of three mice injected with cells with insert 8 died. Three of three mice injected with 10^6 LD842(9-18Δ7::Tn*1725*, numbers 2, 3, 5, and 8) cells died. Two of three mice injected with cells with inserts 1, 4, and 7 died, and one of three injected with insert 6 died.

*Bal*31 digests demonstrated that a 33-kb plasmid that preserved the *Sal*I site to the right of the unique *Xba*I site and to the left of the Tn*5* insert remained virulent ($LD_{50} < 18$ bacteria), while a digest that resulted in a 31-kb plasmid and led to the loss of the left *Sal*I site but conservation of the right site was only partially virulent (LD_{50} of 1.7×10^3 bacteria), confirming the data produced by transposon mutagenesis (Fig. 1). Hence, nine distinct regions of the plasmid 9-18Δ7 were found to be necessary for virulence. Particularly well defined by transposon mutagenesis are a 4.1-kb *Eco*RI fragment and an adjacent 2.4-kb *Eco*RI-*Sal*I fragment.

Seventeen of the inserts which affected virulence were mapped to a 4.1-kb *Eco*RI fragment (Fig. 1). This *Eco*RI fragment appears to be highly conserved among serotypes virulent for mice, including *S. cholerasuis* and *S. typhimurium* (19). Krause et al. have determined the nucleotide sequence of this region and found three open reading frames coding for proteins with predicted molecular weights of 65,500, 28,100, and 27,600 (14). These three proteins appear to be similar in size to those expressed from the 4.1-kb *Eco*RI fragment of *S. cholerasuis* with molecular weights of 29,000, 32,000, and 70,000 (15). Two proteins of 28 and 71 kDa were also reported as being produced in *E. coli* maxicells from a 5-kb *Eco*RI-*Hpa*I fragment of the plasmid of *S. typhimurium* (18) which shares homology with the 4.1-kb fragment of *S. dublin*.

Four inserts which affect virulence are located on a 2.4-kb *Eco*RI-*Sal*I fragment which is adjacent to the 4.1-kb *Eco*RI fragment. Inserts which do not affect virulence are located on either side of these inserts and are approximately 1.5 kb apart. The nucleotide sequence of the *Eco*RI-*Sal*I fragment has also been determined, and a single open reading frame coding for a protein with a molecular mass of approximately 28 kDa was found (14). This protein has not been expressed.

Outside of these two contiguous fragments accounting for 6.5 kb of 9-18Δ7, eight inserts that inactivated virulence, as defined by survival of three of three mice following intraperitoneal injection of 10^3 bacteria, were found. Upon further testing for attenuation, plasmids with these inserts appeared to confer partial virulence to *S. dublin* LD842, as defined by death of at least one of three mice injected with 10^6 bacteria.

Adherence and invasion of epithelial cells by *Salmonella* cells appears to be chromosomally mediated (8). The synthesis of over 30 *Salmonella* proteins is selectively induced during infection of macrophages (3). One of these proteins, PhoP, is chromosomally mediated and is essential for intracellular survival and resistance to defensins (6). The relative contribution of the plasmid to the ability of a strain to cause systemic disease varies with different serotypes. *S. typhimurium* is less affected by removal of its virulence plasmid than is *S. dublin* or *Salmonella enteritidis* (22). The plasmid of *S. dublin* appears to be essential to establish a progressive systemic infection (10). A recent study suggests that a virulence plasmid-cured strain of *S. dublin* exhibited shorter O-specific side chains than its parent strain (13). Hence, it is possible that the plasmid of *S. dublin* confers virulence through alteration of the constituents of the outer membrane. *Bal*31 digests and mutagenesis with Tn*1725* suggest that at least nine distinct regions of the plasmid 9-18Δ7 are involved in the expression of virulence and that, in our study, the smallest deletion derivative conferring full virulence is 33 kb in size.

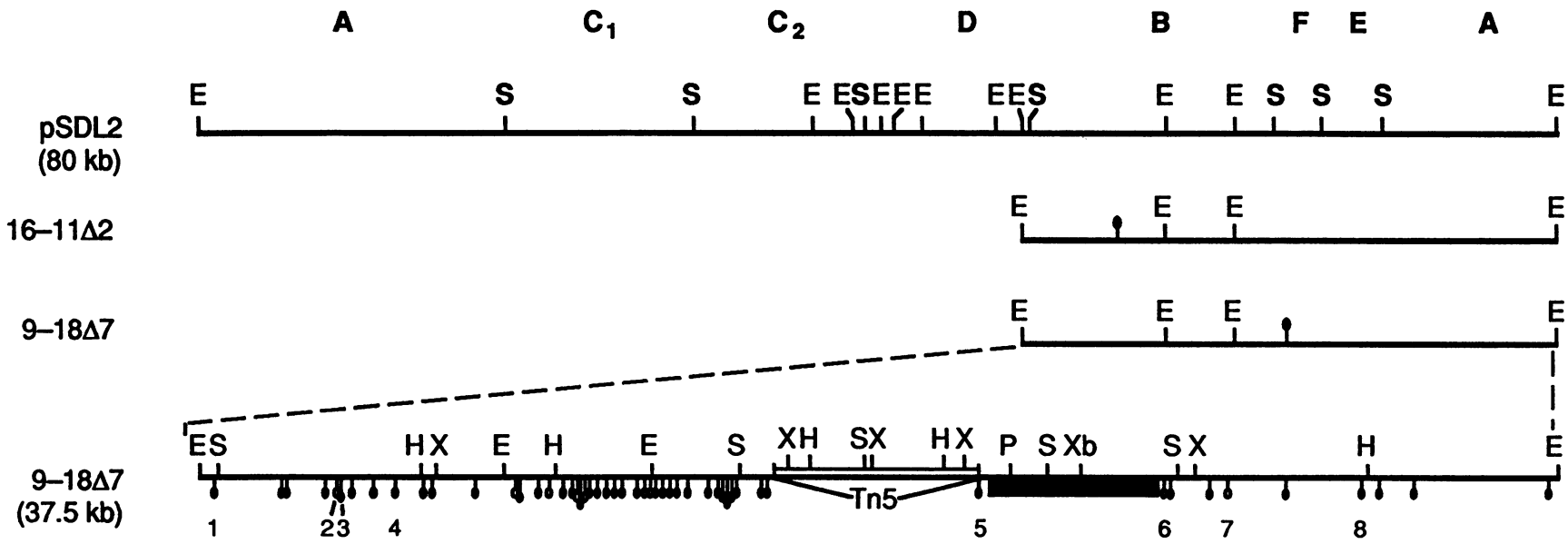

FIG. 1. Effect of Tn*1725* mutagenesis on virulence of 9-18Δ7. Endonuclease restriction map of pSDL2 and of derivatives 16-11Δ2 and 9-18Δ7. Closed ovals above the line indicate Tn*5-oriT*. 9-18Δ7 underwent transposon mutagenesis with Tn*1725*. Closed ovals below the line indicate Tn*1725* inserts found not to affect virulence [as defined by death of three of three mice injected intraperitoneally with 10^3 *S. dublin*(9-18Δ7::Tn*1725*) cells], and open ovals below the line indicate inserts found to affect virulence (as defined by survival of three of three mice). The closed box indicates the region deleted from 16-11Δ2 by *Bal*31 nuclease without affecting virulence. Restriction enzymes: E, *Eco*RI; H, *Hin*dIII; P, *Pvu*I; S, *Sal*I; Xb, *Xba*I; X, *Xho*I.

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