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Characterization of Translation Termination Mutations in the *spv* Operon of the *Salmonella* Virulence Plasmid pSDL2

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The *spv* region of the *Salmonella* virulence plasmids consists of five genes located on an 8-kb fragment previously shown to be essential for virulence in mice. Four structural genes, *spvABCD*, form an operon that is transcriptionally activated by the *spvR* gene product in the stationary phase of growth. The role of the individual *spv* genes in the virulence phenotype was tested by isolating translation termination linker insertions in each gene. Analysis of proteins synthesized in minicells identified each of the *spvABCD* gene products and confirmed the dependence of *spv* structural gene expression on the SpvR regulatory protein. The oligonucleotide insertions in *spvA*, *-B*, and *-C* were shown to be nonpolar. Virulence testing indicated that the SpvB protein, regulated by SpvR, is essential for *Salmonella dublin* to cause lethal disease in mice. Inserts in *spvC* and *spvD* were unstable in vivo for unknown reasons, but these mutants still killed mice at slightly higher inocula. Abolition of *spvA* had no effect on virulence in this system.

The nontyphoid *Salmonella* serovars *S. typhimurium*, *S. choleraesuis*, *S. dublin*, *S. enteritidis*, *S. gallinarum*, and *S. pullorum* harbor large plasmids required for the production of lethal, systemic disease in experimental animals (1, 2, 5, 8, 10, 16–18, 25, 45). For the 80-kb *S. dublin* plasmid, the essential virulence genes are clustered on an 8-kb *Sal*I-*Xho*I fragment (22, 48). Analysis of the nucleotide sequence of this region revealed six open reading frames arranged in the same orientation (22). Hybridization and sequencing studies have demonstrated very similar virulence regions on plasmids from the other serovars, and functional studies have shown that the mouse virulence phenotype encoded by the plasmids is interchangeable between serovars (1–3, 9, 22, 26, 27, 30–32, 35, 36, 38, 41, 43, 47). The first five open reading frames have been recently designated *spv* genes, in recognition of the allelic relationship of these loci from different serovars (12). The sixth open reading frame, *vsdF* from pSDL2, is not essential for mouse virulence (22).

The first gene in the core virulence region, *spvR*, encodes a transcriptional activator belonging to the MetR-LysR family of bacterial regulatory proteins (4, 7, 15, 34, 44). SpvR acts in *trans* as a growth-dependent regulator to turn on expression of the remaining *spv* genes as the bacteria enter the stationary phase (7, 20). Expression of the downstream *spvA* through *-D* genes depends on a promoter region located immediately upstream from *spvA*, and mRNA analysis indicates that these genes form an operon despite the relatively long intergenic distances (20).

Several groups have isolated transposon insertions that abolish virulence and that map within the *spv* region (3, 9, 11, 19, 23, 29, 37, 42). However, the transcriptional organization of the *spvABCD* genes as an operon activated by *spvR* suggests that these transposon mutations do not allow analysis of the phenotypes of individual genes because of polar effects. For this reason, we used a translational termination linker to create nonpolar mutations in each of the *spv* genes. This analysis demonstrates that the *spvR* and *spvB* loci are essential for virulence in mice. Protein analysis in minicells confirms the gene products of the *spvA*, *-B*, and *-C* genes and identifies the *spvD*-encoded polypeptide. Only the *spvR* gene appears to be involved in regulation of *spv* gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Table 1 describes the strains and plasmids used in this study. The transfer-proficient helper plasmid pRK2073 was maintained in *Escherichia coli* C600 Δ*trpE5 recA*. Luria-Bertani (LB) broth and agar were used for routine cultures; Mueller-Hinton (BBL) medium containing trimethoprim (100 μg/ml) was used for selection of pRK2073. Bacteria were grown in Trypticase soy broth containing penicillin (250 μg/ml) for the mouse inoculations. M9 medium supplemented with 0.1 μg of nicotinamide per ml was used for nutritional selection (28).

Recombinant DNA methods. Plasmid DNA isolation, restriction analysis, gel electrophoresis, ligations, and transformations were performed by standard procedures (24).

Mutagenesis. Random oligonucleotide linker mutagenesis of pCR4 was done with a synthetic 14-mer containing an *Xba*I site and translational stop codons in all three reading frames (dCTAGTCTAGACTAG; New England Biolabs). Random single cuts in pCR4 were obtained by limited digestion with dilute pancreatic DNase in Tris-MnCl$_2$ buffer (14). pCR4 DNA (50 μg) was treated with 2.4 pg of pancreatic DNase I (Stratagene) for 20 min at 24°C. The reaction was performed in 300 μl of 20 mM Tris-HCl–1.5 mM MnCl$_2$ in the presence of serum albumin (100 μg/ml). The unit-length linear plasmid molecules were purified by agarose gel electrophoresis and electroelution. After treatment with T4 DNA polymerase to ensure blunt ends, the linear molecules were ligated to the linkers (1:30 molar ratio) with T4 DNA ligase. Excess unligated linker was eliminated by selective precipitation of plasmid DNA (6% polyethylene glycol 6000, 0.6 M NaCl, final concentration). The nicked circular form of the plasmid was obtained by heating the mix to 65°C in 0.15 M NaCl and cooling to 4°C for 1 h (33). The preparation was

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference
S. dublin Lane	Wild-type pSDL2-containing isolate	5
S. dublin LD842	Cured of pSDL2	5
E. coli JA221	*leuB* Δ*trpE5 lacY recA hsdR hsdM*$^+$	3
E. coli χ925	*E. coli* K-12 P678-54 *thr ara leu* Azi^r *lonA lacY* T6^8 *minA gal minB* Shr^r *nalA xyl mtl thi sup*	6
pTJS124	RK2 replicon, Am^r	22
pCR4	*Sal* B fragment of pSDL2 cloned into pTJS124	22
pUD2	7.5-kb *Eco*RI-*Sal*I deletion of pCR4	22
pRK2073	ColE1 replicon, Tra$_{RK2}$, Tm^r	5

transformed into *E. coli* JA221 with selection for penicillin resistance. Plasmid DNA from resistant clones was screened by *Xba*I digestion, and unit-length molecules containing a single *Xba*I site within the 14-kb *Sal*I fragment of pCR4 were selected for further study. Insertion mutations mapping within the *spv* region were located precisely by DNA sequencing. Additional site-specific mutations were obtained by insertion of the *Xba*I linker into a known restriction site by a similar procedure, except that the specific restriction enzyme was used instead of DNase for the initial digestion.

DNA sequencing. The exact site of the *Xba*I linker insertion was obtained by cloning *Xba*I-*Eco*RI and *Xba*I-*Hin*dIII fragments of the mutant plasmids into M13mp19 and M13mp18, respectively. The DNA sequence was determined by the dideoxynucleotide chain termination method (39).

In vitro plasmid stability. The mutant pCR4 derivatives were transferred into the plasmid-free *S. dublin* LD842 by the triparental mating procedure with pRK2073 as the helper (5, 22). Trimethoprim-sensitive transconjugants were selected to ensure the absence of the helper plasmid in LD842. Stability of the mutant pCR4 plasmids in LD842 was tested by growth in LB broth without antibiotic selection for 30 generations. The cells were diluted and replica plated on LB agar with and without penicillin.

Virulence studies in mice. The virulence of the pCR4 mutants was tested by intraperitoneal inoculation of BALB/c mice as described previously (3, 13). Briefly, the LD842 strains containing pCR4 mutants were grown overnight in Trypticase soy broth containing penicillin. After dilution in normal saline to approximately 10^4 CFU/ml, 0.1 ml of cells was injected intraperitoneally. Groups of three to five female mice were used for each mutant in a given experiment, and LD842 strains with and without pCR4 served as controls. A mutant was considered virulent if the number of mice dying by 10 days was comparable to the group inoculated with pCR4. None of the LD842-infected mice died with this inoculum. Surviving mice were sacrificed on day 14, and their spleens were homogenized and cultured quantitatively on Trypticase soy agar with or without penicillin. Mutants were considered avirulent if the spleen counts on day 14 were comparable to the LD842 controls. Plasmid stability of the mutants during infection in vivo was confirmed by comparing the colony counts with or without penicillin. DNA rearrangements in the avirulent mutants were excluded by extraction of the plasmids from colonies obtained from the spleens and analysis by restriction digests. The virulence phenotype for each mutant was confirmed by repeated testing on separate occasions.

Protein synthesis in minicells. Selected pCR4 mutants were transformed into the minicell-producing *E. coli* χ925 (6). A 200-ml culture of *E. coli* χ925 containing a pCR4 mutant was grown for 15 h to reach stationary phase in brain heart infusion medium containing penicillin. Large whole cells were removed by low-speed centrifugation. Minicells were purified through two sucrose gradients. After purification, the minicells were resuspended in 50 to 200 μl of M9-based minimal medium supplemented with methionine assay medium (Difco) at a concentration of approximately 2×10^9 to 4×10^9 minicells per ml and preincubated at 37°C, with shaking, for 20 min. [^{35}S]methionine (10 μCi) was added, and labelling was performed at 37°C for 1 h. The labelled minicells were pelleted by centrifugation and resuspended in 20 to 80 μl of sodium dodecyl sulfate (SDS) dissociating buffer containing β-mercaptoethanol. Synthesized proteins were analyzed by SDS-10 or -12% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. Molecular weights were determined by using Rainbow (Amersham) markers.

RESULTS

Isolation of translation termination linker insertions in the *spv* region. The recombinant plasmid pCR4, containing the *Sal*I B fragment of the *S. dublin* virulence plasmid pSDL2, restores the mouse virulence phenotype to the plasmid-cured *S. dublin* LD842 (22). We constructed linker insertions in pCR4 by partial DNase I digestion in the presence of Mn^{2+} to generate linear molecules, and this was followed by ligation to a 14-mer oligonucleotide containing an *Xba*I site and translation termination codons in all three reading frames. Figure 1 shows the location of 12 linker insertions obtained by DNase I digestion and mapping within the *Sal*I-*Xho*I portion of the *Sal*I B fragment containing the *spv* genes. The exact locations of all the insertions were con-

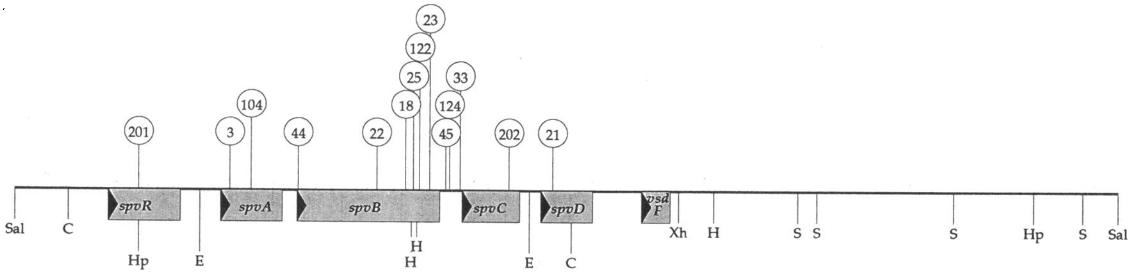

FIG. 1. Map of oligonucleotide linker insertions in the *Sal*I B fragment of pSDL2 cloned on pCR4. Numbers in circles show the locations of the unique inserts. Abbreviations: Sal, *Sal*I; C, *Cla*I; Hp, *Hpa*I; E, *Eco*RI; H, *Hin*dIII; Xh, *Xho*I; and S, *Sma*I.

TABLE 2. Position of linker insertions and virulence[a]

Insert	Position (bp)	Locus	Virulence
201	1539 (*Hpa*I)	*spvR*	−
3	2681	*spvA*	+
104	2945	*spvA*	+
44	3531	*spvB*	−
22	4508	*spvB*	−
18	4870	*spvB*	−
25	4966	*spvB*	−
122	5044	*spvB*	−
23	5169	*spvB*	−
45	5371	Intergenic *spvBC*	+
124	5417	Intergenic *spvBC*	+
33	5500	Intergenic *spvBC*	+
202	6158 (*Tth*111I)	*spvC*	(+)
21	6709	*spvD*	(+)

[a] Virulence was determined by intraperitoneal inoculation of BALB/c mice with approximately 10^3 bacteria, except inserts 202 and 21, for which 10^4 organisms were given. +, virulence comparable to pCR4; −, avirulence comparable to the plasmid-free strain; and (+), death of all the mice at the higher inoculum used.

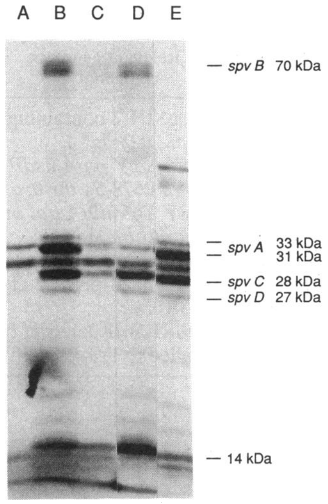

FIG. 2. SDS-PAGE analysis of proteins synthesized in minicells by pCR4 and selected *spvR*, *spvA*, and *spvB* mutants. Lanes: A, pTJS124 control; B, pCR4, C, *spvR201*; D, *spvA104*; E, *spvB22*.

firmed by DNA sequencing and are given in Table 2 according to the nucleotide sequence of Krause et al. (22), beginning at the *Sal*I site upstream from *spvR*. The DNase I-generated mutations were located in *spvA* (mutants 3 and 104), *spvB* (mutants 44, 22, 18, 25, 122, and 23), the intergenic region between *spvB* and *-C* (mutants 45, 124, and 33), and in *spvD* (mutant 21). To obtain mutations in the *spvR* and *spvC* genes, we constructed site-specific insertions of the linker into the *Hpa*I site (mutant 201) and the *Tth*111I site (mutant 202).

Virulence of *spv* mutants. Each pCR4 plasmid containing a linker mutation was transferred into the plasmid-free *S. dublin* LD842 for virulence testing, and the results are shown in Table 2. The single mutant containing an insertion in *spvR*, located at the *Hpa*I site in the N-terminal half of the protein, was avirulent. Two mutations are located in *spvA*, *spvA3* near the N terminus and *spvA104* near the middle of the gene. Both *spvA* mutants exhibited normal virulence on the initial screening test (Table 2) and remained virulent on retesting in a direct comparison with pCR4 and pSDL2 (Table 3). Six mutations in *spvB* were isolated and characterized, spanning the gene from the N terminus (*spvB44*) to the C terminus (*spvB23*). All the *spvB* mutants were nonvirulent, including insertions close to the C-terminal end. Three mutations mapped in the intergenic region between *spvB* and *spvC*, and these mutants were virulent. The *spvC202* mutant, constructed in the *Tth*111I site, was virulent when given at a slightly higher inoculum (10^4). At a lower inoculum, plasmid instability in vivo was observed, making virulence testing difficult to interpret. A similar problem with in vivo stability occurred with the *spvD21* mutant, which also was virulent at the higher inoculum.

Expression of proteins by the *spv* locus in minicells. To examine the effects of the *spv* linker mutations on protein expression, the peptides synthesized by these plasmids in minicells were labeled with [^{35}S]methionine and resolved on SDS-PAGE. The proteins synthesized by pCR4 are shown in Fig. 2, lane B, and compared with the vector control (lane A). Under these conditions, pCR4 expresses nine polypeptides from the cloned *Sal*I B fragment of pSDL2: a 70-kDa doublet (SpvB), 33- and 31-kDa peptides (SpvA), a 28-kDa peptide (SpvC), a 27-kDa peptide (SpvD), and smaller peptides of 24, 21, 16, and 14 kDa (the latter may represent the *vsdF* gene product as described below). The SpvR protein was not identified in these gels, and mRNA analysis indicates that *spvR* is expressed at low levels in the wild-type plasmid (19a). The effects of the *spvR201* mutation are shown in Fig. 2, lane C. Marked decreases in the SpvA, -B, -C, and -D proteins are seen. However, the *spvA104* mutation (Fig. 2, lane D) specifically affects only the SpvA protein and not the downstream genes. A truncated SpvA′ protein is seen at 14 kDa, as predicted from the location of the termination insertion (Fig. 1). The *spvB22* mutant, shown in Fig. 2, lane E, produces the predicted truncated B′ protein at 37 kDa, but does not affect downstream gene expression (*spvC* and *-D*) or *spvA*.

TABLE 3. Virulence of *spvA* mutants

Plasmid	Inoculum ($\log_{10}$)	No. of deaths/total mice
pCR4	3.5	2/3
pSDL2	3.7	3/3
pCR4*spvA3*	2.9	2/3
pCR4*spvA104*	3.9	2/3

The effects of insertion mutations in *spvC* and *spvD* are shown in Fig. 3. The pCR4 control is in lane A. The *spvC202* mutant (lane B) shows the 25-kDa truncated protein (predicted to be 22 kDa from the sequence) instead of the wild-type SpvC. No changes in the amounts of the other *spv* gene products are seen, including the downstream SpvD protein. However, both insertion of the linker in *spvD21* (lane D) and deletion of the *spvD* coding sequence (lane C) lead to loss of the 27-kDa SpvD protein band. Lane C also shows loss of a conspicuous band at 14 kDa, the presumptive gene product of *vsdF*, an open reading frame downstream of *spvD*. Constitutive synthesis of mRNA from *vsdF* has been reported previously (7).

DISCUSSION

The isolation of nonpolar translation termination insertions in the *spvA*, *-B*, *-C*, and *-D* genes, coupled with corresponding protein expression gels, confirms the previous sequence results predicting the existence of these genes

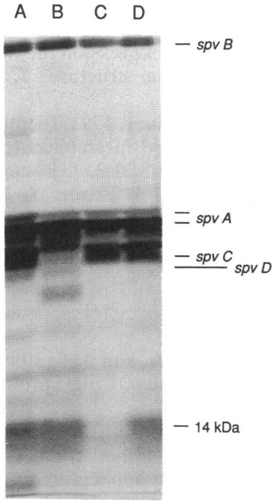

FIG. 3. SDS-PAGE analysis of proteins synthesized in minicells by *spvC* and *spvD* mutants. Lanes: A, pCR4; B, *spvC202*; C, pUD2 (deletion of the 7.5-kb *Eco*RI-*Sal*I fragment containing *spvD*, *vsdF*, and the remaining rightward portion of the *Sal*I B fragment shown in Fig. 1); D, *spvD21*.

on the *S. dublin* virulence plasmid pSDL2 (22). Overexpression of the SpvA, -B, and -C proteins in *S. dublin* has recently been reported (46), and the identity of the SpvA, -B, and -C proteins has also been demonstrated by N-terminal sequencing of the overproduced proteins from *S. typhimurium* (40). However, expression from the native promoter was not shown in either study. The SpvD protein was predicted from the DNA sequence and the results of deletion analysis demonstrating a role for the *spvD* region in virulence (22). The present study identifies the SpvD protein and also tentatively assigns the 14-kDa protein encoded by pCR4 to the *vsdF* gene, located downstream of *spvD*.

Although the SpvR protein was not seen on these gels showing expression from the intact virulence region, this protein has been identified from an overproducing clone (40). *spvR*-specific mRNA is present in relatively low levels in cells containing the native pSDL2 plasmid, indicating low levels of expression consistent with its role as a positive regulatory protein (7, 19a). Genetic data clearly indicate that SpvR acts in *trans* to activate *spvABCD* expression (4, 7, 20). Transcription of the operon initiates at two closely linked promoter sites upstream of *spvA*, and this region is required for expression of the four downstream structural genes (20). These findings are dramatically confirmed by the protein expression of the *spvR201* mutant (Fig. 2, lane C), showing marked decreases in SpvA, -B, -C, and -D synthesis.

Transcription analysis by Northern (RNA) blotting as well as genetic evidence indicates that the *spvABCD* genes form an operon (20). Previously isolated transposon insertions in the *spv* locus are likely to have exerted polar effects on the expression of downstream genes (3, 9, 11, 19, 23, 29, 37, 42). Therefore, the virulence phenotypes reported for these mutants may not accurately reflect the roles of the individual *spv* genes. Our approach involved both random and site-specific insertions of a synthetic oligonucleotide containing translation termination codons in all three reading frames. This 14-mer linker is unlikely to affect transcription, and the considerable intergenic distances between *spv* genes make translational coupling between the reading frames unlikely. These predictions were confirmed by the experimental findings showing that insertion of the linker into each *spv* structural gene coding sequence had no effect on the expression of the other Spv proteins. These results also indicate that only SpvR appears to have a major regulatory role in the *spvABCD* operon and that none of the other Spv proteins are required for *spv* gene expression.

Virulence testing of the mutants showed that *spvR* and *spvB* are required to produce lethal, systemic infections in mice. The *spvR201* linker insertion mutant confirms previous results with a Tn*5*-*oriT* insertion (15-2) in pSDL2 close to the N terminus of *spvR* (3, 22) and similar mutations in the *S. typhimurium* plasmid (4, 23, 36, 44). The mechanism of virulence attenuation is likely to be due to lack of expression of the *spvABCD* operon. All the insertion mutants obtained in *spvB* were avirulent, including mutations close to the C terminus of the gene. The primary structure of SpvB is remarkable for a long run of prolines (nine in *S. dublin*, seven in *S. typhimurium*) close to the middle of the molecule that may serve to separate the higher-order structure of the protein into different domains (22). The linker insertion mutants suggest that the entire SpvB protein is required for expression of the virulence phenotype.

In contrast, the use of nonpolar termination mutations demonstrates that SpvA is not essential for virulence in the BALB/c mouse model. *spvA104* produces a truncated SpvA polypeptide, while *spvA3*, close to the N terminus, completely abolishes SpvA. However, both mutants display virulence comparable to that of pCR4. The role of *spvA* in wild-type infections of natural hosts remains to be determined.

The *spvC202* and *spvD21* mutations, important for demonstrating the protein products of these genes, were less useful for virulence testing because of instability in vivo. The mechanism of this destabilization is not clear, since both constructs still contain the multimer resolution function encoded by the region downstream of the *spv* locus on the *Sal*I B fragment (21, 22). Clearly, both mutations retain at least a partial virulence phenotype. A mutant with a Tn*5*-*oriT* insertion at the start of *spvC* (15-1) in pSDL2 is avirulent (3, 22), and a Tn*5* insert in the intergenic region upstream of *spvC* in *S. typhimurium* likewise resulted in decreased virulence (9). However, polar effects of these insertions may have affected both *spvC* and *spvD* together. The cloned *spvC* gene, not under its normal transcriptional control, partially restores virulence to a plasmid-cured *S. typhimurium* strain, and the C-terminal region was shown to be important for this phenotype (9). Since the insert in *spvC202* occurs upstream from this essential region, this mutation is expected to abolish the normal function of SpvC. The fact that the *spvC202* mutant remains virulent at an inoculum of 10^4 indicates that the *spvC* activity reported by Gulig and Chiodo (9) for *S. typhimurium* does not have nearly as prominent a role as the *spvB* gene in *S. dublin*. Deletion of the 1.8-kb *Eco*RI-*Xho*I fragment containing *spvD* and *vsdF* led to partially attenuated virulence in an artificial pUD2-2 construct (22). Since Tn*5*-*oriT* insertions in *vsdF* remain virulent, these results were interpreted to suggest an accessory virulence role for *spvD*. The present data show that the predicted SpvD protein is produced under the control of *spvR* and are consistent with a secondary virulence role. In summary, the evidence clearly demonstrates the primary importance of *spvB*, regulated by the *spvR* gene product, in the expression of mouse virulence in *Salmonella* species. Accessory virulence functions are likely for *spvC* and *spvD*, while *spvA* is not essential in this system.

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