Virulence region of plasmid pNL2001 of *Salmonella enteritidis*

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The virulence region of the *Salmonella enteritidis* 55 kb plasmid pNL2001 was identified by Tn1-insertion mutagenesis, DNA hybridization studies, and Western blot analysis of proteins encoded in the virulence region of the plasmid. DNA hybridization studies showed that the pNL2001 plasmid contained a 6·4 kb *Sall*–*EcoRI* fragment homologous to the 6·4 kb *Sall*–*EcoRI* *Salmonella* plasmid virulence (*spv*) region of the *S. choleraesuis* 50 kb plasmid (pKDSC50). One of the 247 Tn1-insertion mutants of *S. enteritidis*, designated strain TA19, showed a reduced mouse lethality, and the Tn1-insertion of strain TA19 was located within this homologous 6·4 kb region, suggesting that the 6·4 kb *Sall*–*EcoRI* fragment of pNL2001 contained the *spv* region. Two contiguous *Sall*–*EcoRI* (2·3 kb) and *EcoRI*–*EcoRI* (4·1 kb) fragments derived from the 6·4 kb *Sall*–*EcoRI* fragment were cloned into the expression vectors. By Western blot analysis using four Spv peptide antisera, each specific for individual proteins encoded in the *spvR*, *spvA*, *spvB* and *spvC* genes of pKDSC50, four proteins encoded in the 6·4 kb *Sall*–*EcoRI* fragment of pNL2001 were identified. Protein SpvR with an apparent molecular mass of 32 kDa was produced from the 2·3 kb *Sall*–*EcoRI* fragment, and proteins SpvA, SpvB and SpvC with apparent molecular masses of 32, 70 and 29 kDa, respectively, were produced from the 4·1 kb *EcoRI*–*EcoRI* fragment. From the 4·1 kb *EcoRI*::Tn1 fragment of the TA19 plasmid, proteins SpvA and SpvB were expressed, but not SpvC. It was therefore suggested that the *spvC* gene may contribute to the expression of virulence of *S. enteritidis*. Furthermore, the nucleotide sequence of the 6·4 kb *Sall*–*EcoRI* fragment encoding these four proteins was determined. Four open reading frames which encoded the four proteins with deduced molecular masses of 33 906, 28 200, 65 349 and 27 646 Da were detected. Deduced amino acid sequences of each protein showed a high degree of identity to corresponding sequences in the virulence region of *S. choleraesuis*, *S. dublin* and *S. typhimurium* virulence plasmids. Therefore, we confirmed that the virulence plasmids of *Salmonellae* including *S. enteritidis* share the highly conserved region responsible for virulence.

Keywords: *Salmonella enteritidis*, virulence plasmid pNL2001, *spvABC* genes

INTRODUCTION

Salmonella enterica subsp. *enteritidis* causes enteric and systemic infection in man and animals. *S. enteritidis* contains the serotype-specific large plasmid which is

responsible for virulence in mice (Nakamura *et al.*, 1985; Hovi *et al.*, 1988; Suzuki *et al.*, 1989), as well as other non-typhoidal *Salmonellae* (Gulig, 1990). These virulence-associated plasmids primarily contribute to the systemic infection of organisms beyond the intestine to the mesenteric lymph nodes, spleen and liver in mice (Gulig & Curtiss, 1987; Heffernan *et al.*, 1987; Suzuki *et al.*, 1992). The identification of the virulence region of the plasmids is important to analyse the effect of the plasmid

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on mouse virulence. The virulence regions of the *S. typhimurium*, *S. dublin* and *S. choleraesuis* plasmids have been defined by deletion analysis, transposon mutagenesis or gene cloning (Baird *et al.*, 1985; Michiels *et al.*, 1987; Williamson *et al.*, 1988b; Gulig, 1990; Kawahara *et al.*, 1990). DNA sequence analysis of these virulence regions indicates that approximately 8.0 kb virulence regions of the *S. typhimurium*, *S. dublin* and *S. choleraesuis* plasmids are almost identical to each other, and encode at least five genes, *spvRABCD*, which express proteins of 25–66 kDa (Norel *et al.*, 1989a, b, c; Pullinger *et al.*, 1989; Taira & Rhen, 1989a, b, 1990; Gulig & Chiodo, 1990; Matsui *et al.*, 1990a, b, 1993; Krause *et al.*, 1991; Caldwell & Gulig, 1991; Gulig *et al.*, 1992, 1993).

Based on DNA hybridization studies and introduction of virulence plasmids into the different serotypes of *Salmonella*, it was predicted that the virulence plasmids of *S. typhimurium*, *S. dublin*, *S. choleraesuis* and *S. enteritidis* shared the homologous region associated with the virulence (Popoff *et al.*, 1984; Beninger *et al.*, 1988; Williamson *et al.*, 1988a; Korpela *et al.*, 1989; Poppe *et al.*, 1989; Woodward *et al.*, 1989; Roudier *et al.*, 1990; Jones & Osborne, 1991). Based on the results of electron microscopic heteroduplex analysis of the virulence plasmids from different serotypes, Montenegro *et al.* (1991) have reported recently that *S. typhimurium*, *S. dublin*, *S. choleraesuis* and *S. enteritidis* plasmids share large regions of homology. Although Williamson *et al.* (1988a) transduced a transposon insertion from the virulence region of the *S. typhimurium* and *S. dublin* plasmid to the *S. enteritidis* plasmid and showed that the resulting strains were avirulent in mice, the precise location of the virulence region of the *S. enteritidis* plasmid has not been identified. As a result, the genetic characterization of the virulence region of the *S. enteritidis* plasmid is poorly documented.

In this report, we describe the physical and genetic characterization of the *S. enteritidis* virulence plasmid pNL2001. We constructed a restriction endonuclease cleavage map of pNL2001 and found a 6.4 kb region encoding the virulence phenotype. Sequence analysis of this region revealed four sequential open reading frames encoding putative proteins of 29–70 kDa.

METHODS

Bacterial strains, plasmids, bacteriophages and media. *S. enteritidis* AL1190 contained the 55 kb (36 MDa) virulence plasmid pNL2001, and AL1192 was a plasmid-cured derivative of AL1190 (Nakamura *et al.*, 1985). *S. choleraesuis* RF-1 (Kawahara *et al.*, 1988), which contained the 50 kb virulence plasmid pKDSC50, was used for DNA hybridization studies and preparation of DNA probes. *Escherichia coli* TH471 (Harayama *et al.*, 1980) was used for transposon mutagenesis of pNL2001 as the transposon Tn1 carrier. *E. coli* HB101 (Bolivar & Backman, 1979) and JM109 (Yanisch-Perron *et al.*, 1985) were used as host strains for recombinant plasmids. pACYC184 (Chang & Cohen, 1978), pUC118 and pUC119 (Viera & Messing, 1987), and pHSG398 and pHSG399 (Takeshita *et al.*, 1987) were used as cloning vectors. The nucleotide sequencing vectors were M13mp18 (Yanisch-Perron *et al.*, 1985) and Bluescript M13 (Short *et al.*, 1988). pMKD201 and pMKD401 carried the 4.1 kb *EcoRI*–*EcoRI* fragment (*spvA*, *spvB* and *spvC*

genes) of pKDSC50 into pUC118 and the 2.3 kb *SalI*–*EcoRI* fragment (*spvR* gene) of pKDSC50 into pUC119, respectively (Matsui *et al.*, 1991). The growth media used were Antibiotic Medium 3 (Difco), Heart Infusion broth (Eiken), L broth (Lennox, 1955), L agar containing 1.5% (w/v) agar, and desoxycholate–hydrogen sulfide–lactose agar (DHL; Eiken). Antibiotics were added for selection at the following concentrations: ampicillin (Ap), 25 µg ml⁻¹; kanamycin (Km), 25 µg ml⁻¹; chloramphenicol (Cp), 25 µg ml⁻¹, and tetracycline (Tc), 12.5 µg ml⁻¹.

Preparation of restriction map. After timed partial digestion of the 55 kb plasmid pNL2001 with the restriction enzymes *SalI*, *EcoRI* or *HindIII*, digested fragments were cloned into pACYC184 and resulting recombinant plasmids were transferred to *E. coli* HB101 by transformation. The recombinant plasmid DNAs were isolated, digested, and analysed by 0.7–1.0% (w/v) agarose gel electrophoresis.

Transposon mutagenesis of pNL2001 with Tn1. Insertion mutagenesis using Tn1 was performed as described previously (Nakamura *et al.*, 1985), employing pTH10 as the transposon carrier. *S. enteritidis* AL1190 was mated with the donor strain *E. coli* TH471, which carries the pTH10 plasmid. Ap^r and Km^r *Salmonella* exconjugants were selected in glucose minimal medium containing Ap and Km, and then cultured at 42 °C to select for the transposition of Tn1 and the curing of pTH10. Resulting Ap^r and Km^s colonies were selected by replica plating, and plasmid DNA from these colonies was transferred into the plasmid-cured derivative AL1192 by the procedure of Kushner (1978) to exclude strains in which Tn1 had been inserted into the chromosome. These transformants were designated Tn1 inserts.

Mouse infection. Female ddY mice, 5 weeks old, were purchased from Nippon SLC. To screen for the strains carrying the plasmid in which Tn1 had been inserted into the virulence region, approximately 10⁵ c.f.u. of cultures (0.1 ml) of Tn1 inserts were subcutaneously injected into the abdomen of three mice. Seven days after inoculation, 0.1 ml blood was collected by puncture of the retro-orbital venous plexus, inoculated in 3 ml of Heart Infusion broth, and incubated at 37 °C overnight. The overnight cultures were subcultured on DHL agar containing Ap at 37 °C overnight. Mice were monitored for 14 d and deaths were recorded. The strain which was not recovered from the blood of mice and did not cause the death of inoculated mice was considered to be avirulent, as well as plasmid-cured strain AL1192. The others were considered to be virulent, as well as wild-type strain AL1190.

To determine the LD₅₀ value of each strain, groups of five mice were inoculated with 10-fold serial dilutions of cultures subcutaneously into the abdomen or intraperitoneally, and deaths were recorded over 14 d. LD₅₀ values were calculated by the method of Behrens–Kärber (Kärber, 1931).

Genetic methods. Isolation of plasmid DNAs from *Salmonella* strains and from *E. coli* was performed by the method of Kado & Liu (1981), and by the method of Holmes & Quigley (1981), respectively. Purification of plasmid DNA was carried out by caesium chloride–ethidium bromide gradient ultracentrifugation. Transformation of plasmid DNA was performed as described by Kushner (1978) and Cohen *et al.* (1972). Restriction enzyme digestion, ligation and other molecular genetic manipulations were performed with enzymes purchased from Takara Shuzo or Toyobo, and were used according to the instructions supplied by the manufacturers. Digested DNA was electrophoresed at 100 V on 0.7–1.0% (w/v) agarose gel prepared in TBE buffer (40 mM Tris base, 20 mM sodium borate, 1 mM EDTA, pH 8.2).

(a)

(b)

Fig. 1. (a) Restriction endonuclease map of the virulence plasmid pNL2001 of *S. enteritidis*. *spv* represents the region associated with virulence in mouse. (b) Construction of the recombinant plasmids. Horizontal arrows and arrowhead indicate the *lacZ* promoter of the cloning vectors and *Tn1* insertion site of strain TA19, respectively. Restriction endonuclease sites are as follows: A, *AatI*; B, *BamHI*; E, *EcoRI*; H, *HindIII*; P, *PstI*; S, *SalI*.

Southern blot analysis. Plasmid DNAs pNL2001 and pKDSC50 were digested with *SalI*, *HindIII* and *EcoRI*, individually, or *EcoRI* and *SalI* together. Digested plasmid DNAs were fractionated on 1% (w/v) agarose gel, and transferred onto a nylon membrane (Hybond-N+; Amersham). The probes from the 2.3 kb *SalI*–*EcoRI* fragment and 4.1 kb *EcoRI*–*EcoRI* fragment of pKDSC50 were labelled with horseradish peroxidase. Hybridization on nylon membrane and detection of chemiluminescent signals on X-ray film (Hyperfilm-ECL, Amersham) were performed using ECL direct nucleic acid labelling and detection systems (Amersham) according to the manufacturer's instructions.

Table 1. Mouse lethality of an avirulent mutant of *S. enteritidis*

Mice were inoculated subcutaneously (SC) or intraperitoneally (IP).

Strain	Plasmid	log LD ₅₀ (c.f.u.)	
		SC	IP
TA19	pNL2001::Tn1	7.7	5.1
AL1192	–	8.1	6.1
AL1190	pNL2001	5.6	< 2.4

Antisera against individual Spv proteins. Antisera for Western blot analysis were prepared as described previously (Matsui *et al.*, 1993). Briefly, oligopeptides were synthesized based on the amino acid sequence of hydrophilic regions near the C-termini of the SpvR, SpvA, SpvB and SpvC proteins encoded in the *spv* region of pKDSC50. The synthetic peptide sequences were as follows: SpvR, YPTKKRETPDYRKAIKLIQC; SpvA, SNY-AMELASRLSPEQQTLPTPDNS; SpvB, SKLRLSDDAT-ADTNRIKRIC; SpvC, YRNELRSGRDGGEMQRQALRE-EPFYPLMTE. Individual antisera against Spv proteins were generated by immunization of rabbits with synthetic peptide-bovine serum albumin conjugates of corresponding proteins in Freund's complete adjuvant. Each serum exhibited a monospecificity based on examinations by ELISA (Matsui *et al.*, 1993) and Western blot analysis using the system described below (data not shown), although some cross-reactivity was reported between the SpvR and SpvB proteins using these antisera (Matsui *et al.*, 1993).

Western blot analysis. *E. coli* JM109 carrying the recombinant plasmid was cultured overnight in the presence of IPTG at a final concentration of 1 mM. SDS-PAGE was performed according to the method of Laemmli (1970). The proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose paper (Bio-Rad). Non-specific antibody binding was blocked by incubating the nitrocellulose in 3% (w/v) gelatin and 5% (v/v) horse serum in TBS buffer [20 mM Tris (pH 7.5), 500 mM NaCl, 0.01% (w/v) merthiolate]. The blots were probed with rabbit antiserum against the Spv protein. Binding of the primary antibody was detected by using goat anti-rabbit immunoglobulin G conjugated to horse radish peroxidase (HRP; Cappel) and then by incubating the preparation in HRP colour development reagent (containing 4-chloro-naphthol) (Bio-Rad). *E. coli* JM109 carrying pMKD401 or pMKD201 were used as positive controls expressing the SpvR protein (32 kDa) and SpvA (32 kDa), SpvB (70 kDa) and SpvC (29 kDa) proteins, respectively. *E. coli* JM109 carrying the vector plasmid was used as a negative control.

Nucleotide sequencing. The 2.3 kb *SalI*–*EcoRI* and 4.1 kb *EcoRI*–*EcoRI* fragments of pNL2001 were cloned into Bluescript M13 and M13mp18, respectively. Nucleotide sequencing was performed with an automatic 373A DNA sequencer using the *Taq* Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystem). Sequencing primers were provided in the kit. All the sequences were read from both strands. DNA sequences flanking *Tn1* insertions were determined by using oligonucleotides corresponding to the termini of *Tn3* (Heffron *et al.*, 1979) as primers. The sequence data were analysed with DNA software, GENETYX-MAC (Software Development Co.).

Fig. 2. Southern hybridization analysis of pNL2001 with 2.3 kb *SalI*-*EcoRI* (a) and 4.1 kb *EcoRI*-*EcoRI* (b) probes derived from pKDSC50. pNL2001 (lanes 1-4) and pKDSC50 (lanes 5-8) were digested with *SalI* (lanes 1 and 5), *SalI* plus *EcoRI* (lanes 2 and 6), *HindIII* (lanes 3 and 7) and *EcoRI* (lanes 4 and 8). *HindIII* fragments of λ DNA were used as molecular mass standards.

Fig. 3. Western blot analysis of Spv proteins expressed from recombinant plasmids. Lanes: 1, JM109/pMKD401; 2, JM109/pMKD201; 3, JM109/pUC118; 4, JM109/pUC119; 5, JM109/pNE135; 6, JM109/pNE100; 7, JM109/pNEG19; 8, JM109/pNEG21; 9, JM109/pHSG398; and 10, JM109/pHSG399. Spv peptide antisera used were as follows: anti-SpvR serum (a), anti-SpvA serum (b), anti-SpvB serum (c) and anti-SpvC serum (d).

RESULTS

Endonuclease restriction map

In the agarose gel electrophoretic patterns of pNL2001 digested with *SalI*, *EcoRI* and *HindIII*, there were 8 *SalI* sites, 11 *EcoRI* sites (the first and third bands of the *EcoRI*

digest were doublets), and 7 *HindIII* sites (the second band of the *HindIII* digest was a doublet). With the timed partial digest of pNL2001 with each enzyme, the restriction map of pNL2001 was constructed as shown in Fig. 1. Compared with the restriction enzyme sites, the maps of pNL2001 and pKDSC50 (Kawahara *et al.*, 1990)

<i>Sal</i> I	
1	GTCGACTTCATCCTGATAAAACACAGGATGTGCAGTCTGCTCCTGAGCCAGCGCCTGATCAATGACAAGCCGTTTTCTT 80
81	CATAGTGCGGATCTTTGATTTTCAGCGTTGGCGCTGCCCTGCCAGACCATATCTGCCTGCCTCAGATATCGGTGCAGG 160
161	GTGGAGCGGTGCAGCGTCACCAAAAGTCGGTTGATGACGAGTACCAGAAGCTCCGTGCTCCAGCGTGAGCGCAGCATCA 240
241	GCCAAAATCCTTCGGGGAACGCTGTACCAATAGTGGCAAGAGCTGAAGGATATCAGCGACCGGCCAGCGTGGAGCACGTC 320
321	CGGGTCTGAGGCTTTTTAGTCTTCAACACCATGTAAGTAAACCAGTTTATCCATCGACCAACGGATGAACGGCGGCA 400
401	CAGAGTAATCTGGCAACGTCAGTGACGGTCATTCCCTGATGCAGCATCAGCATGGCGATAAGCCGTCGCGCATGGTTTT 480
481	ATCGTAGGTTTGTGGGCTCTTGGCGATCAGTTGTCGTTCTTCATCAGGAATTGCTGCTATGATCGGCATCGCTCAGT 560
561	CCGGTTGGTGATTTGTGATGTTTGGCGATTGATCAGATCGACAATCCGGGCTGAGTTCCTTTTCAGTGATCTACTATT 640
641	TGCGAAGCTATTTAGTGACACTAATCGATTTTTCAGACAACCTTTCTCGCCTGGCGTGAGTTTTCGTTTCAGTGAACCT 720
721	ATAAAAGGCTCTGCATTCTGCTGTAGTGCGCCAGGCCTAATAACGCTCCCGTTTAATTCGGGCCGGTCGTACATC 800
801	GGTTCGTTCATTTATGGGATCGGACTTTTTCGTTGTAATGCTTCAAGAGCGATTTTTGTTCTGCGGTCAACTCAACA 880
881	TTATAGCCATCAGCATGATCCTGATTATTTTGAACCAAGCATCTTCATTGATCATGGGTATACATCGTTGTTATCCA 960
961	GCTATACATCATAACAGGTCAATTAATCCACTCAGAAATAAGTCAGGGTATGCATAAAATCTATCGCCATAATCCTA 1040
1041	TCCAGTAACACCATGATTAGTAAGAATAATCAGTCTGTGCAAAAACAGGTCAACGCCATCCTGTTTTGCACATCAAAA 1120
→ <i>spvR</i> -35	
1121	CATTTTTTCAGGATTATCTGAAAAAAAGGAGATATTATGGATTTCTTGATTAATAAAAAATTTTTCATAAC 1200
1	ACTGATGGAACAGGTTCTTTCAGTATCGCAACATCAGTACTGTATATACCCGAACCCGCTGAGCAGGGTTATTTAG 14
1201	ACTGATGGAACAGGTTCTTTCAGTATCGCAACATCAGTACTGTATATACCCGAACCCGCTGAGCAGGGTTATTTAG 1280
15	L M E T G S F S I A T S V L Y I T R T P L S R V I S D 41
1281	ACCTGGAAAGAGAGCTGAAACAAAGACTCTTTATACGGAAGAATGGCACTCTTATCCCAACCGAATTTGCACAACTATT 1360
42	L E R E L K Q R L F I R K N G T L I P T E F A Q T I 67
1361	TATCGAAAAGTAAATCCATTATTTTCTTACATGCACTGGAGCAGGAAATCGGACCTACGGGTAAAACGAAACAAC 1440
68	Y R K V K S H Y I F L H A L E Q E I G P T G K T K Q L 94
1441	AGAAATAATATTTGACGAAATTTATCCGGAAGTTTAAAAATCTGATCATTTTCAGCACTGACCATTTCCGGCCAAAAA 1520
95	E I I F D E I Y P E S L K N L I I S A L T I S G Q K T 121
1521	CAATATAATGGGAGAGCCGTTAACAGCCAAATAATAGAAGAACTGTGTCAGACAAACAACGATTGTTATTTCTGCC 1600
122	N I M G R A V N S Q I I E E L C Q T N N C I V I S A 147
1601	AGAAATATTTTCATCGGAATCGCTTGTCTGCCGGACATCAGTGGAGGGTGGGGTCATGTTATTTATCTAAAAAATT 1680
148	R N Y F H R E S L V C R T S V E G G V M L F I P K K F 174
1681	CTTTCTCTGCGGCAACCTGATATCAACAGGCTGGCCGGAACACCTGTACTTTTTCATGAGGGGGTAAAAATTTAATC 1760
175	F L C G K P D I N R L A G T P V L F H E G A K N F N L 201
1761	TGGACACCATATACATTTTTTAAACAGACACTAGGTATTACCAACCTGCATTTCAGTTTGTAAACGTCGATTGTTC 1840
202	D T I Y H F F K Q T L G I T N P A F S F D N V D L F 227
1841	AGTTCACTGTACCGGTTACAACAAGGGCTGGCGATGTTACTCATCCCGTCAGAGTCTGTGCGGCTCTGGGATTATCAAC 1920
228	S S L Y R L Q Q G L A M L L I P V R V C R A L G L S T 254
1921	AGATCAGCACTGCACATCAAAGCGTAGCGCTCTGTACCTCCTGTATTACCCGACCAAGAAACGGGAGACACCAGATT 2000
255	D H A L H I K G V A L C T S L Y Y P T K K R E T P D Y 281
2001	ATCGTAAAGCTATAAACTGATACAGCAGGAACTGAAACAGTCCACCTTCTGACCTTATGCAGCGTAAGGGCCGAACAC 2080
282	R K A I K L I Q Q E L K Q S T F * 297
2081	CTGTATTCACGGCATTGCGCAGATTGATGTCAGCAATCCCATCCTCCATAGCGGTAGTTCACCGCGGAGCATGGAG 2160
2161	TAAACCGGCTGGTCGCGTCAATCTGACACAGAATCAGTTTGATGCTGCTGGTGATTACCTAAAACATGGGCATTAACGC 2240
<i>Eco</i> RI	
2241	GCTGGCTCAGCCACTTTACTGAAGAACTGAATAACGGTGACTATGACGGCGCAGCGAATGAATT 2306

Fig. 4. Nucleotide sequence of the *spvR* region. The DNA sequence of the 2.3 kb *Sal*I-*Eco*RI region of pNL2001 and deduced amino acid sequence of the coding region are indicated. The horizontal arrows indicate inverted repeats and the asterisk indicate the stop codon, respectively. Potential promoter (-35 and -10) and Shine-Dalgarno (S.D.) sequences are also marked.

resembled each other, especially in the region encompassing the *spv* region.

Tn1-insertion mutants

To identify the *S. enteritidis* virulence region of pNL2001, transposon insertion mutagenesis with Tn1 was carried

out, and 247 insertion mutants were obtained from *S. enteritidis* AL1190. In a screening for avirulent mutants, only one of them, designated strain TA19, induced neither bacteraemia nor death in mice. The LD₅₀ of strain TA19 was almost the same as that of strain AL1192, whilst strain AL1190 showed a lower LD₅₀ (Table 1). Based on restriction enzyme analysis of plasmid DNA from strain

<i>Eco</i> RI	
1	GAATTCCTGAAATGGGACCACGCCAGCGGTGAGTTGTTCCCGGCTGACCCGACGCCGGAGCGCTGAACGTTGTTTATT 80
81	CCTGAGTTAATTTGTTGTGCCATCTTTCACACCGGGAACCGGATTCCGCACAGCAGAAAAATAGCACATAAATAAACT 160
<i>Pst</i> I	
161	CAATATAAGCCACTCATTTTCTGGCAATACAAAATAATTCCCTGCAGACATTATCAGTCTTCAGGATTTTCATTCTGTTT 240
→ <i>spvA</i>	
241	ATTTTCAAGGATCATCATTATTTATGAATATGAATCAGACCACCACTCCGGCACTTTCACAGGTCGAAACCGCCATCCGG 320
1	S. D. M N M N Q T T S P A L S Q V E T A I R 19
321	GTCCCGCAGGGAATTTTGCAAAATATAATTATTATTCGGTGTGATATTGTCGTGAGACCCGTAACAGTTTATTAA 400
20	V P A G N F A K Y N Y Y S V F D I V R Q T R K Q F I N 46
401	CGCCAATATGTCATGGCCGGGCTCCCGGAGGTAAAGCCTGGGACCTGGCGATGGGCCAGGCGAGTATATCCGCTGCA 480
47	A N M S W P G S R G G K A W D L A M G Q A Q Y I R C M 73
481	TGTTCCGAGAAAAATCAATTGACCCGAGAGTTTCGGGGACCTTGACAGAGACTGGACAATGGCAGAACCTGAGCAGT 560
74	F R E N O L T R R V R G T L Q Q T L D N G T N L S S 99
561	TCCGCTGTCCGGGTATTCAGGGACAGGCAGAGCGTCCGGCCGACCTGGCCACCTGATGGTGGTTAATGATGCCATTAA 640
100	S A V G G I Q G Q A E R R P D L A T L M V V N D A I N 126
641	CCAGCAATACCGACCCCTGCTGCGTATCATTTTCCACAGCAGGTTGAGTTATCTCTGCTGAATACCGATGTGTCCG 720
127	Q Q I P T L L P Y H F P H D Q V E L S L L N T D V S L 153
721	TGGAAGATATTATCAGCGAGAGCAGCATTGACTGGCGTGGTTCCTGAGCAACTCGCTGACCGGCGATAACAGTAACAT 800
154	E D I I S E S S I D W P W F L S N S L T G D N S N Y 179
801	GCCATGGAGCTCGCCAGCCGGCTGTCAACAGAGCAGCAGACTGCCGACCGAGCCGACAACAGTACCGCCACTGACCT 880
180	A M E L A S R L S P E Q Q T L P T E P D N S T A T D L 206
881	GACCTCTTTTTACAGACCAATCTGGGGCTGAAAACCGCCGACTATACGCCATTTGAAGCACTGAATACCTTTGCCCGAC 960
207	T S F Y Q T N L G L K T A D Y T P F E A L N T F A R Q 233
961	AGTTAGCGATTACCGTCCCCCAGGTGGAACAGTTGATTGCGGGTACTCTGCGTGCCAGCCGGCAGTTTAGCTTCCCGCG 1040
234	L A I T V P P G G T V D C G Y S A C Q P A V * 255
<i>Pst</i> I	
1041	CTACCAGAGTAGTGAGCAGCAGACATTCTGCAGAATCTGAGCGACGTATTGTTCAAGTGCACTTCTACCGCGCTGTACG 1120
1121	GCGGCAGCACTTTTGAACAGGCCGTAGAGCAGACGCTGTAAGCAGAAAAATACCTGGCCATCGTCAGACGGCCAGTTTC 1200
→ <i>spvB</i>	
1201	AGGAGATAGTGTATGTTGATACTAAATGGTITTTTCATCTGCCACTTTAGCGCTGATCACTCCCCCTTTCTGCCAAAAGG 1280
1	S. D. M L I L N G F S S A T L A L I T P P F L P K G 23
1281	GGGCAAGCGCTGAGTCAGTCAGGCCCTGACGGCCTAGCCAGTATAACGCTGCCTCTGCCATCAGCGCCGAACGCGGCT 1360
24	G K A L S Q S G P D G L A S I T L P L P I S A E R G F 50
1361	TTGCGCTGCGTGGCGTGCCTACAGCAGCGGTGGCGGAATGGCCCTTCGGCGTGGGCTGGTCTGCGGACAATG 1440
51	A P A L A L H Y S S G G G N G P F G V G W S C A T M 76
1441	AGCATTGCCCGCAGCAGCCATGGCGTGGCGCAGTATAACGACAGCGATGAGTTTCTGGGGCCGACGGAGAAGTGCT 1520
77	S I A R S T S H G V P Q Y N D S D E F L G P D G E V L 103
1521	GGTTCAAACGCTCAGCACCAGTATGCCCCAATCCCGTCACTGCTTCGCGTACGGTGACGTATCGTTCCCGCAAAGCT 1600
104	V Q T L S T G D A P N P V T C F A Y G D V S F P Q S Y 130
1601	ACACGGTGACCCGCTATCAGCCCCGACGGAGAGCAGTTTATCGCTGGAGTACTGGGTGGGCAACAGCAACGGCGAT 1680
131	T V T R Y Q P R T E S S F Y R L E Y W V G N S N G D 156
1681	GATTTCTGGTTACTGCATGACAGTAACGGCATCCTGCACCTGCTGGGAAAAACCGCCGACGACGCTCAGCGATCCGCA 1760
157	D F W L L H D S N G I L H L L G K T A A A R L S D P Q 183
1761	GGCCGCTCTCATACGGCGCAATGGCTGGTTGAGGAGTCGGTGACCCCTGCCGCGAGCATATCTATTACTCTACCTGG 1840
184	A A S H T A Q W L V E E S V T P A G E H I Y Y S Y L A 210
1841	CGGAGAACGGTGACAATGTGGACCTCAATGGGAACGAGCGCGGACGCGATCGCAGCGCATCGCTATCTCAGCAAGGTA 1920
211	E N G D N V D L N G N E A G R D R S A M R Y L S K V 236
1921	CAGTATGCCAACGCGACCCCGCGCGGATCTGTACCTCTGGACTAGCGCCACACCCGCGGTACAGTGGCTGTTCACCCT 2000
237	Q Y G N A T P A A D L Y L W T S A T P A V Q W L F T L 263
2001	AGTGTTTGACTACGGCGAACGTGGTGTAGATCCACAGGTACCGCTGCATTCACTGCTCAGAACAGCTGGCTCGCCCGCC 2080
264	V F D Y G E R G V D P Q V P P A F T A Q N S W L A R Q 290
<i>Bam</i> HI	
2081	AGGATCCCTTCTCCCTGTATAACTACGGCTTTGAGATCCGCCTCCATCGCTGTGCCGCCAAGTCCTGATGTTCCACCAC 2160
291	D P F S L Y N Y G F E I R L H R L C R Q V L M F H H 316
2161	TTTCCTGATGAACTGGGTGAAGCGATACGCTGGTTTCCCGTCTGCTGCTGGAGTATGACGAAAAATCCGATACTGACACA 2240
317	F P D E L G E A D T L V S R L L L E Y D E N P I L T Q 343

2241	GCTTTGCGCTGCTCGGACGCTGGCCTATGAAGGCGACGGTTATAGAAGAGCTCCTGTCAACAATATGATGCCACCGCCAC	2320
344	L C A A R T L A Y E G D G Y R R A P V N N M M P P P P	370
2321	CGCCTCCTCCGATGATGGGAGGTAATTCATCTCGACCAAAATCAAAATGGGCGATTGTAGAGGAATCAAAGCAGATTCAA	2400
371	P P P M M G G N S S R P K S K W A I V E E S K Q I Q	396
2401	GCTCTGAGGTACTATTCAAGGTACAGTGTGATTAATAAATATTTACGTGGGGATGATTATCCTGAAACACAGGC	2480
397	A L R Y Y S A Q G Y S V I N K Y L R G D D Y P E T Q A	423
2481	AAAAGAACTCTGCTCTCCAGAGACTATCTTTCCACAAATGAACCCAGTGATGAGGAGTTTAAAAATGCCATGTCAGTTT	2560
424	K E T L L S R D Y L S T N E P S D E E F K N A M S V Y	450
	<i>Hind</i> III	
2561	ATATAAATGATATTGCGGAGGGATTAAGTTCACCTCCCGAAACAGATCACAGAGTCGTATACCGGGGCGCTGAAGCTTGAT	2640
451	I N D I A E G L S S L P E T D H R V V Y R G L K L D	476
	<i>Hind</i> III	
2641	AAGCCCGCATTATCGGATGTGCTGAAGGAATACACTACTATAGGTAATATAATAATAGATAAAGCTTTTATGAGTACATC	2720
477	K P A L S D V L K E Y T T I G N I I I D K A F M S T S	503
2721	GCCAGATAAGGCATGGATAAATGACACTATTCTCAACATATACCTAGAAAAAGGACATAAAGGTAGAACTACGCGAGATG	2800
504	P D K A W I N D T I L N I Y L E K G H K G R I L G D V	530
	<i>Bam</i> HI	
2801	TTGCACATTTTAAGGGAGAGGCAGAGATGCTTTTCCCTCCAAATACTAAACTCAAATCGAAAGCATTGTAAATTGTGGA	2880
531	A H F K G E A E M L F P P N T K L K I E S I V N C G	556
2881	TCCCAAGACTTTGCAAGCCAGCTTAGTAAGCTGAGATTAAGTGATGCAACTGCTGACACAAACAGGATAAAAAGAAT	2960
557	S Q D F A S Q L S K L R L S D D A T A D T N R I K R I	583
2961	AATAAATGAGGGTACTCAACTCATAGATACTAAGAATCTATTCCAGAAGTGGTATGAGCGGCCTAGCTCTATAAGGGG	3040
584	I N M R V L N S *	591
3041	TTATACTCCGGAACCCAGATTTTCCGTACCCCTAGGCCCGCAAAGTAGTGCCATCTAACTTTTGCCATTACCCCTTCTT	3120
3121	TAACTTTCTGCTCGGAACGGACCGAAATATCATTTTTTCGCTGATAAAAATGAGGTTTTCTGGATAACTAATCGTTTT	3200
	<i>spvC</i>	
3201	ATTAATAAATACTGAGAATTTATATCTAATAATATGGCGATATATCCATATCGCAAGGAGATTTCCCATGCCATAAAT	3280
1	<i>Aat</i> I -35 -10 S.D. M P I N	4
3281	AGGCCTAATCTAAATCTAAACATCCCTCCTTTGAATATTGTAGCTGCTTATGATGGGCGGAAATACCATCTACAAATAA	3360
5	R P N L N L N I P P L N I V A A Y D G A E I P S T N K	31
3361	GCACCTGAAAAATAATTTCAACTCCTTGCAACAACCAATGCGGAAGATGCCGGTATCCCACTTTAAGAGGGCGCTGGATG	3440
32	H L K N N F N S L H N Q M R K M P V S H F K E A L D V	58
3441	TGCTGACTATTCAAGGATGCCCGAGAGTGGTTTCTTTGCTATGAGCCAAGGTTTTGAGTGAATAACCATGGTTACGAT	3520
59	P D Y S G M R Q S G F F A M S Q G F Q L N N H G Y D	84
3521	GTTTTATCCATGCTCGTGCAGAAATCACCTCAGTCTCAGGGCAAATTTGCCGGTGACAAGTTCACATCAGTGTGCTCAG	3600
85	V F I H A R R E S P Q S Q G K F A G D K F H I S V L R	111
	↓	
3601	GGATATGGTGCCACAAGCATTTCAGCGCTGTCCGATTGCTGTTTTAGAGGACAGTCCGGTAGATAAGTGGAAGTGA	3680
112	D M V P Q A F Q A L S G L L F S E D S P V D K W K V T	138
3681	CCGATATGGAGAAGGTCGTTCAACAAGCCCGTGTAGCCTGGGCGCTCAGTTCACGTTGTATATAAAACCAGACCAGGAA	3760
139	D M E K V V Q Q A R V S L G A Q F T L Y I K P D Q E	164
3761	AATTCGCAGTACAGTGCCTCGTTTCTCCACAAGACACGGCAATTTATAGAGTGTCTGGAATCCAGACTATCCGAAAATGG	3840
165	N S Q Y S A S F L H K T R Q F I E C L E S R L S E N G	191
3841	GGTTATTTCAAGGACAGTGTCTGAGTCAGACGTTTCATCCTGAAAATTGGAATATCTCAGTTATCGTAATGAACACTACGAA	3920
192	V I S G Q C P E S D V H P E N W K Y L S Y R N E L R S	218
3921	GTGGCGTGATGGTGGTGAATGCAGAGACAGGCTTTACGTGAGGAACCGTTTTATCGTTTGATGACAGAGTAAGTATGG	4000
219	G R D G G E M Q R Q A L R E E P F Y R L M T E *	241
4001	GTTTGGGAGCAACGGAACAGTAAACGCCGTAAACAGCTATTTAAATGCTCATTATTTATTAATCAATAAATTACAA	4080
	<i>Eco</i> RI	
4081	ATTTTCATTGAAGGCTCCCCCTTACTGACGAATTC	4116

Fig. 5. Nucleotide sequence of the *spvA*–*spvC* region. The DNA sequence of the 4.1 kb *Eco*RI–*Eco*RI region of pNL2001 and deduced amino acid sequences of the coding regions are indicated. The horizontal arrows, asterisk, and arrowhead indicate inverted repeats, the stop codon, and *Tn7* insertion site of strain TA19, respectively. Potential promoter (–35 and –10) and Shine–Dalgarno (S.D.) sequences are also marked.

TA19, the Tn1-insertion site was detected in the 1.4 kb region of the *Hind*III–*Eco*RI fragment of the plasmid (indicated by the arrowhead in Fig. 1).

Southern blot analysis

To identify the homologous virulence region in pNL2001, the 2.3 kb *Sa*I–*Eco*RI fragment of *S. choleraesuis* plasmid pKDSC50, containing the *spvR* gene, and the 4.1 kb *Eco*RI–*Eco*RI fragment, containing the *spvA*, *spvB* and *spvC* genes, were used as probes for Southern blot analysis. As shown in Fig. 2(a) and (b), the 2.3 kb and 4.1 kb probes hybridized with the 2.3 kb *Sa*I–*Eco*RI and 4.1 kb *Eco*RI–*Eco*RI (*Eco*RI-6) fragments of the 55 kb plasmid pNL2001 of *S. enteritidis*, respectively. The 2.3 kb probe also hybridized with the *Sa*I-1, *Eco*RI-1 and *Hind*III-1 fragments in the restriction enzyme map of pNL2001 (Fig. 1). Accordingly, it was demonstrated that the 2.3 kb *Sa*I–*Eco*RI fragment was contiguous to the *Eco*RI-6 fragment, and that the 6.4 kb *Sa*I–*Eco*RI fragment homologous to the *spv* region of pKDSC50 was present in pNL2001.

Identification of proteins encoded by the virulence region

To identify the proteins encoded in the 6.4 kb *Sa*I–*Eco*RI fragment of pNL2001, *E. coli* JM109 carrying the various recombinant plasmids (Fig. 1b) were analysed by Western blotting using four antisera against individual SpvR, SpvA, SpvB and SpvC proteins expressed from the 6.4 kb *spv* region of the *S. choleraesuis* plasmid pKDSC50. The recombinant plasmids were constructed using pUC118, pUC119, pHSG398 or pHSG399 as cloning vectors. Results of Western blot analysis are shown in Fig. 3. Anti-SpvR serum enabled detection of the 32 kDa protein in JM109/pMKD401 used as a positive control for SpvR, but not in JM109/pMKD201 (data not shown). Anti-SpvA, -SpvB and -SpvC sera enabled detection of the 32, 70 and 29 kDa proteins in JM109/pMKD201, respectively, but not in JM109/pMKD401 (data not shown). None of the proteins were detected in the JM109 strains carrying the vector plasmid. One protein, designated SpvR with a molecular mass of 32 kDa, was detected in cells carrying pNE135 or pNE130, and three proteins of 70, 32 and 29 kDa, were detected in cells carrying pNE100 or pNEG21. To confirm the order of the proteins expressed in the 4.1 kb *Eco*RI–*Eco*RI fragment of pNL2001, 0.9 kb *Pst*I–*Pst*I, 2.2 kb *Pst*I–*Aat*I and 1.2 kb *Bam*HI–*Eco*RI fragments from the 4.1 kb *Eco*RI–*Eco*RI fragment were subcloned into the vector plasmids pUC119 or pUC118. pNE141 and pNE151 were constructed by insertion of the *Pst*I–*Aat*I fragment from pNE100 into the *Pst*I–*Sma*I site of pUC119 and pUC118, respectively. The 70 kDa protein was detected in JM109/pNE141, the 32 kDa protein in JM109/pNE142, and the 29 kDa protein in JM109/pNE192 and JM109/pNE182, indicating that the order of the molecular mass of the proteins designated SpvA, SpvB, and SpvC from left to right along the fragment was 32, 70 and

29 kDa, respectively (Fig. 1a). No proteins were detected in JM109/pNE132 or JM109/pNE151, indicating that the translational orientations of the SpvA and SpvB proteins were as shown in Fig. 1(a) along the 4.1 kb *Eco*RI–*Eco*RI fragment. SpvR and SpvC proteins were detected in JM109/pNE130 and JM109/pNE182, respectively, suggesting the presence of a promoter sequence in each fragment.

The *Eco*RI-6::Tn1 fragment of the TA19 plasmid was cloned into the vector plasmid pHSG398, designated pNEG19, and proteins expressed from pNEG19 were examined. From pNEG19, SpvA and SpvB were expressed, unlike SpvC. No truncated protein was detected (Fig. 3, lane 7).

Determination of DNA sequences of the region involved in *S. enteritidis* virulence

The DNA sequence of the 6.4 kb *Sa*I–*Eco*RI fragment of pNL2001 was determined (Figs 4 and 5). Four open reading frames consisting of 891, 765, 1773 and 723 bp were identified from nucleotides 1160–2050 at the *Sa*I site of the 2.3 kb *Sa*I–*Eco*RI fragment (Fig. 4), and from nucleotides 264–1028, 1213–2985 and 3269–3991 at the left *Eco*RI site of the 4.1 kb *Eco*RI–*Eco*RI fragment (Fig. 5), respectively. These open reading frames encoded peptides with deduced molecular masses of 33 906, 28 200, 65 349 and 27 646 Da, which were in good agreement with the sizes estimated from Western blot analysis.

The consensus ribosome-binding sites (AGGAG) were identified in a region 6–13 bp upstream of the ATG start codon of each open reading frame. The consensus promoters, –10 (GATTAT, TATATC) and –35 sequences (TGCACA, TTTATA), were identified upstream of two open reading frames encoding SpvR and SpvC (Figs 4 and 5).

The DNA sequences and deduced amino acid sequences of the *spv* region of *S. enteritidis* were compared with the corresponding genes and proteins of the *S. typhimurium*, *S. dublin* and *S. choleraesuis* plasmids reported previously (Norel *et al.*, 1989a, b, c; Pullinger *et al.*, 1989; Taira & Rhen, 1989a, b, 1990; Gulig & Chiodo, 1990; Matsui *et al.*, 1990a, b, 1993; Krause *et al.*, 1991; Caldwell & Gulig, 1991; Gulig *et al.*, 1992). There were no significant differences in the nucleotide sequences in the coding regions between the *spv* region from *S. enteritidis* and the other three serotypes. In the *spvR* and *spvA* regions, substitutions of 2 and 1–3 nucleotides were observed, resulting in 2 and 1–3 amino acid alterations, respectively. In the *spvB* and *spvC* regions, substitutions of 3–9 and 0–3 nucleotides were observed, respectively, except for the sequences described by Norel *et al.* (1989a, b), resulting in 1–4 and 0–3 amino acid alterations, respectively. As shown in Table 2, the identity of each deduced amino acid sequence in the *spv* region of *S. enteritidis* to the corresponding sequences in the *spv* region of other plasmids exceeded 98% except for the results reported by Norel *et al.* (1989a, b). As cited by Taira & Rhen (1989a) and Gulig *et al.* (1992), some substitutions and deletions

Table 2. Comparison of deduced amino acid sequences of Spv proteins encoded in the *spv* regions of the *S. enteritidis* plasmid with those of other *Salmonella* virulence plasmids

Serotype	Percentage identity to:				Reference(s)
	SpvR	SpvA	SpvB	SpvC	
<i>S. choleraesuis</i>	99.3	99.6	99.3	98.9	Matsui <i>et al.</i> (1990a, b, 1993)
<i>S. dublin</i>	99.3	99.2	99.5	100.0	Krause <i>et al.</i> (1991)
<i>S. typhimurium</i>	99.3	99.2	99.3	100.0	Taira & Rhen (1989a, b, 1990)
	99.3	98.8	94.4	87.1	Norel <i>et al.</i> (1989a, b, c)
	99.3	99.2	99.8	99.6	Caldwell & Gulig (1991), Gulig & Chiodo (1990), Gulig <i>et al.</i> (1992)
	99.3	ND*	ND	ND	Pullinger <i>et al.</i> (1989)

ND, Not determined.

* Unpublished.

of nucleotides exist between the *spvB* and *spvC* regions of *S. enteritidis* and the corresponding regions reported by Norel *et al.* (1989a, b), respectively, resulting in the relatively low percentages of identity (Table 2).

DNA sequence analysis of the Tn1 insertion

To identify the precise location of the Tn1 insertion site of strain TA19, the DNA sequences flanking the Tn1 insertion of pNEG19 were determined. The insertion site was located at base 3647 of the 4.1 kb *EcoRI*-*EcoRI* fragment within the coding sequence of SpvC (Fig. 5).

DISCUSSION

Several authors have reported the presence of a homologous region in the *S. enteritidis* plasmid to the virulence region of the *S. typhimurium* and *S. dublin* plasmids based on DNA hybridization studies (Popoff *et al.*, 1984; Beninger *et al.*, 1988; Williamson *et al.*, 1988a; Korpela *et al.*, 1989; Poppe *et al.*, 1989; Woodward *et al.*, 1989; Roudier *et al.*, 1990; Jones & Osborne, 1991). Recently, heteroduplex analysis of the virulence plasmids of several serotypes has shown that the *S. typhimurium*, *S. choleraesuis* and *S. enteritidis* plasmids were very similar (Montenegro *et al.*, 1991). Moreover, Williamson *et al.* (1988a) demonstrated the avirulence in mice of *S. enteritidis* mutants in which the transposon-containing sequences from the virulence region of the plasmid of *S. typhimurium* M242 or

S. dublin M51 were transduced into the plasmid. Therefore, it has been suggested that the homologous region in the *S. enteritidis* plasmid presumably contributed to the virulence. However, the precise location of the homologous region on the restriction map of the *S. enteritidis* plasmid has not been defined. In the present study, we constructed the restriction enzyme map of the *S. enteritidis* plasmid pNL2001 (Fig. 1a) and showed, based on Southern blotting analysis, that a 6.4 kb *SalI*-*EcoRI* region of the *S. enteritidis* plasmid was homologous to the 6.4 kb *SalI*-*EcoRI* *spv* region of the *S. choleraesuis* plasmid (Fig. 2). One mutant, TA19, in which Tn1 had been inserted within the homologous region, showed reduced mouse lethality (Table 1). Although we could not obtain any other mutants in which Tn1 had been inserted within the homologous region, *S. enteritidis* transductants described by Williamson *et al.* (1988a) were not virulent in mice, and the transposon insertions were located within the 2.3 kb *SalI*-*EcoRI* and 4.1 kb *EcoRI*-*EcoRI* fragments (Baird *et al.*, 1985). On the basis of these results, it was suggested that the homologous region in the *S. enteritidis* plasmid pNL2001 was responsible for virulence in mice. This homologous region was mapped on pNL2001 as the *spv* region of *S. enteritidis* plasmid (Fig. 1a).

Four proteins were detected from the 6.4 kb *spv* region of pNL2001 by Western blotting analysis using individual Spv protein antisera (Fig. 3), and at least four open reading frames corresponding to the individual proteins were identified within the DNA sequence of this region (Figs 4 and 5). We designated these *S. enteritidis* genes *spvR*, *spvA*, *spvB* and *spvC*, encoding 32, 32, 70 and 29 kDa proteins, respectively. The *spv* genes were oriented in the same direction as shown in Fig. 1(a), and corresponded to the sequences of the *spv* genes recently identified in the *S. typhimurium*, *S. dublin* and *S. choleraesuis* virulence plasmids. Comparison of the DNA sequences and deduced amino acid sequences of our *spv* region with those of the other serotypes showed that the *spvR*, *spvA*, *spvB* and *spvC* genes of *S. enteritidis* were nearly identical to the *spvR*, *spvA*, *spvB* and *spvC* genes of the other three serotypes (Norel *et al.*, 1989a, b, c; Pullinger *et al.*, 1989; Taira & Rhen, 1989a, b, 1990; Gulig & Chiodo, 1990; Matsui *et al.*, 1990a, b, 1993; Krause *et al.*, 1991; Caldwell & Gulig, 1991; Gulig *et al.*, 1992), respectively (Table 2). Therefore, it was confirmed that the virulence plasmids of non-typhoidal *Salmonellae*, including *S. enteritidis*, share the highly conserved region responsible for the virulence, although the virulence plasmids carried by different serotypes differed in their size and restriction digest pattern (Popoff *et al.*, 1984). However, in this study, we could not define the region downstream of the *spvC* gene in *S. enteritidis*, where the *spvD* and *orfE* genes were identified in *S. typhimurium* and *S. dublin* (Krause *et al.*, 1991; Gulig *et al.*, 1992, 1993). Considering the almost complete identity of our *spvRABC* genes to those of the other three serotypes and the extensive region of homology among the *Salmonella* virulence plasmids (Montenegro *et al.*, 1991), it is likely that the *spvD* and *orfE* genes are also encoded in *S. enteritidis*, and it would be unsurprising if they are nearly identical to those of the

other serotypes. Identification of these genes in *S. enteritidis* will therefore require further investigation.

Woodward *et al.* (1989) reported that the virulence plasmids of *S. typhimurium*, *S. dublin* and *S. enteritidis* share the approximately 10 MDa homologous region containing the virulence region, and Lax *et al.* (1990) demonstrated the similarity of the two regions, approximately 22 kb encompassing the virulence region and 8 kb outside it, between the *S. dublin* and *S. typhimurium* plasmids. The heteroduplex analysis of *Salmonella* virulence plasmids by Montenegro *et al.* (1991) also showed that the virulence plasmids of *S. typhimurium*, *S. dublin*, *S. choleraesuis* and *S. enteritidis* share large regions of homology. In fact, the restriction enzyme map of the *S. enteritidis* plasmid pNL2001 (Fig. 1a) revealed that the restriction sites of the enzymes on the pNL2001 plasmid, especially on the region encompassing the *spv* region, were very similar to those of the *S. choleraesuis* plasmid pKDSC50 (Kawahara *et al.*, 1990). In a recent study, Selander *et al.* (1992) suggested the evolutionary derivation of *S. dublin* from an *S. enteritidis*-like ancestor based on the similarities of the multilocus enzyme genotype and nucleotide sequence of *fliC* gene between *S. dublin* and *S. enteritidis*. It is thus possible that the *Salmonella* virulence plasmids have conserved a uniform region including the virulence region and have been constructed, serotype-specifically, by the addition or deletion of the non-homologous region through an evolutionary process, as previously suggested by Williamson *et al.* (1988a).

The 29 kDa protein was detected from the subclones JM109/pNE192 and JM109/pNE182, which carried the *Bam*HI–*Eco*RI fragment in opposite orientations against the *lacZ* promoter of the vector plasmid, suggesting the presence of the promoter for the *spvC* gene within the fragment. Based on the DNA sequence analysis, –10 and –35 regions for the consensus promoter were located 23 and 45 bases upstream from the start codon for the *spvC* gene of *S. enteritidis*, respectively. Gulig & Curtiss (1988) and Gulig & Chiodo (1990) suggested the presence of the promoter, which was located approximately 300 bases upstream from the start codon for the *spvC* gene of *S. typhimurium* plasmid. Thus, there is a discrepancy in the location of the consensus promoter sequence between the results reported by Gulig & Chiodo (1990) and ours, although both promoter sequences were detected within each *Bam*HI–*Eco*RI fragment. Most recently, complementation experiments for virulence in mice and for β -galactosidase activity expressed by the *spv*–*lacZ* fusions in *E. coli* and *S. typhimurium* have revealed that the *spvC* gene was transcribed from a promoter located in the 0.4 kb *Bam*HI–*Stu*I fragment, downstream of *spvB* (Coynault *et al.*, 1992). The identification of the derivatives deleted from the *Bam*HI site of the *Bam*HI–*Eco*RI fragment will be necessary to determine the precise location of the promoter for the *spvC* gene.

The function of the products from individual genes within the *spv* region of *S. enteritidis* remains largely unknown. In the present study, we showed that the strain TA19 displayed a reduced mouse lethality, and that Tn1

was located within the *spvC* gene encoding the 29 kDa protein. Complementation examinations confirmed that the *spvC* region of *S. typhimurium* encoding a 28 kDa protein was essential for the expression of virulence in mice (Gulig & Chiodo, 1990; Coynault *et al.*, 1992). However, a non-polar insertion mutation of *spvC* of *S. dublin* retained a partial virulence phenotype for mice, suggesting the presence of the accessory virulence function of the *spvC* gene (Roudier *et al.*, 1992). On the basis of these results and ours, it is suggested that the 29 kDa protein encoded by the *spvC* gene of *S. enteritidis* may contribute to mouse lethality. To confirm the role of our SpvC protein in virulence, it will be necessary to examine the complementation by the *spvC* gene of *S. enteritidis*. Recent studies have demonstrated that the *spvR* gene products of *S. typhimurium*, *S. dublin* and *S. choleraesuis* positively regulated the expression of downstream *spv* genes through the *spvA* promoter (Caldwell & Gulig, 1991; Fang *et al.*, 1991; Krause *et al.*, 1992; Matsui *et al.*, 1991, 1993; Taira *et al.*, 1991; Coynault *et al.*, 1992). The contribution of the *spvR* and *spvD* genes to virulence in mice was also confirmed by recent complementation studies (Gulig *et al.*, 1992; Coynault *et al.*, 1992), whilst the definitive roles of *spvA* and *spvB* genes has been controversial (Gulig *et al.*, 1993). It is assumed that since the sequences of our *spv* genes are almost identical with those of the *spv* genes previously reported, our *spv* genes presumably exert a similar function to that of the *spv* genes in the other three serotypes.

Although it remains to be determined how the virulence gene products contribute to pathogenesis, recent investigations have demonstrated that the *spv* genes were selectively expressed during the stationary phase of culture or under starvation conditions (Fang *et al.*, 1991; Krause *et al.*, 1992; Coynault *et al.*, 1992) and were regulated by a chromosomal gene *katF* which encodes an alternative σ factor of RNA polymerase (Fang *et al.*, 1992; Norel *et al.*, 1992). Rhen *et al.* (1989) reported that Tn5 insertion within the *spvB* region induced a low viability of *S. typhimurium* in murine liver macrophages, indicating that the *spvB* gene was necessary for intracellular growth of bacteria. In contrast, Riikonen *et al.* (1992) reported that the *spv* genes of the *Salmonella* virulence plasmids did not contribute to the survival or growth of *Salmonellae* in murine macrophages cultured *in vitro*. In the most recent study by Gulig & Doyle (1993), it was indicated that the virulence plasmid of *S. typhimurium* contributed to virulence in mice by the increase of growth rate of the bacteria presumably within mouse cells, and not through the induction of resistance to killing by the host or the increase of the mobility of the bacteria between tissues. Therefore, the *spv* gene products are likely to be expressed under regulation of a chromosomal gene *katF* in mouse cells where the nutrient conditions are limited. Thereafter the growth rate of bacteria may increase, resulting in lethality for mice. The higher recovery rates of bacteria from the deeper tissues in mice infected with the parent *S. enteritidis* strain compared with the plasmid-cured derivative described in our previous study (Suzuki *et al.*, 1992) and the reduced mouse lethality of the *spvC* insertion

mutant in the present study may be ascribed to this mechanism.

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