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Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*

(bacterial pathogenesis/pathogenicity island/murine typhoid/secretion/signature-tagged mutagenesis)

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ABSTRACT **Mapping the insertion points of 16 signature-tagged transposon mutants on the *Salmonella typhimurium* chromosome led to the identification of a 40-kb virulence gene cluster at minute 30.7. This locus is conserved among all other *Salmonella* species examined but is not present in a variety of other pathogenic bacteria or in *Escherichia coli* K-12. Nucleotide sequencing of a portion of this locus revealed 11 open reading frames whose predicted proteins encode components of a type III secretion system. To distinguish between this and the type III secretion system encoded by the *inv/spa* invasion locus known to reside on a pathogenicity island, we refer to the *inv/spa* locus as *Salmonella* pathogenicity island (SPI) 1 and the new locus as SPI2. SPI2 has a lower G+C content than that of the remainder of the *Salmonella* genome and is flanked by genes whose products share greater than 90% identity with those of the *E. coli ydhE* and *pykF* genes. Thus SPI2 was probably acquired horizontally by insertion into a region corresponding to that between the *ydhE* and *pykF* genes of *E. coli*. Virulence studies of SPI2 mutants have shown them to be attenuated by at least five orders of magnitude compared with the wild-type strain after oral or intraperitoneal inoculation of mice.**

Salmonella typhimurium is a principal agent of gastroenteritis in humans and produces a systemic illness in mice that serves as a model for human typhoid fever (1). After oral inoculation of mice with *S. typhimurium*, the bacteria pass from the lumen of the small intestine through the intestinal mucosa via enterocytes or M cells of the Peyer's patch follicles (2). The bacteria then invade macrophages and neutrophils, enter the reticuloendothelial system, and disseminate to other organs, including the spleen and liver, where further reproduction results in an overwhelming and fatal bacteremia (3). To invade host cells, to survive and replicate in a variety of physiologically stressful intracellular and extracellular environments, and to circumvent the specific antibacterial activities of the immune system, *S. typhimurium* employs a sophisticated repertoire of virulence factors (4).

To gain a more comprehensive understanding of virulence mechanisms of *S. typhimurium* and other pathogens, we recently developed a transposon mutagenesis system called signature-tagged mutagenesis, which combines the strength of mutational analysis with the ability to follow simultaneously the fate of a large number of different mutants within a single animal (5). By using this approach, we identified 43 mutants with attenuated virulence from a total of 1152 mutants that were screened. The nucleotide sequences of DNA flanking the insertion points of transposons in 5 of these mutants showed that they were related to genes encoding type III secretion systems of a variety of bacterial pathogens (6, 7). The products of the *inv/spa* gene cluster of *S. typhimurium* (8, 9) are proteins that form a type III secretion system required for the assembly of surface appendages mediating entry into epithelial cells (10). Hence, the virulence of strains carrying mutations in the *inv/spa* cluster is attenuated only if the inoculum is administered orally (p.o.) and not when given intraperitoneally (i.p.) (8). In contrast, the 5 mutants identified by signature-tagged mutagenesis are avirulent after inoculation i.p. (5).

In this paper, we show that the transposon insertion points of these 5 mutants and an additional 11 mutants identified by signature-tagged mutagenesis map to the same region of the *S. typhimurium* chromosome. Further analysis of this region revealed additional genes whose deduced products have sequence similarity to other components of type III secretion systems. This region occupies approximately 40 kb at minute 30.7 on the chromosome, is conserved in other *Salmonella* species, but is not present in a number of other enteric bacteria. We have termed this region SPI2 (*Salmonella* pathogenicity island 2) to distinguish it from the SPI (SPI1) at minute 63 (map edition 8; ref. 11), which contains the *inv/spa* cluster (12).

MATERIALS AND METHODS

Bacterial Strains, Transduction, and Growth Media. *Salmonella enterica* serotypes 5791 (*aberdeen*), 423180 (*gallinarum*), 7101 (*cubana*), and 12416 (*typhimurium* LT2) were obtained from the National Collections of Type Cultures, Public Health Laboratory Service, Colindale, U.K. *Salmonella typhi* BRD123 genomic DNA was a gift from G. Dougan (Imperial College, London, U.K.), enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Vibrio cholera* biotype *El Tor, Shigella flexneri* serotype 2, and *Staphylococcus aureus* were clinical isolates obtained from the Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, London. Genomic DNA from *Yersinia pestis* was a gift from J. Heesemann (Max von Pettenkofer-Institut, Munich). The bacterial strains and the methods used to generate signature-tagged mini-Tn*5* transposon mutants of *S. typhimurium* NCTC strain 12023 have been described (5, 13). Routine propagation of plasmids was in *E. coli* DH5α. Bacteria were grown in LB broth (14) supplemented with the appropriate antibiotics. Before virulence levels of individual mutant strains were assessed, the mutations were first transferred by phage P22-mediated transduction (14) to the nalidixic acid-sensitive parental strain of *S. typhimurium* 12023. Transductants were analyzed by restriction endonuclease digestion and Southern blot hybridization before use as inoculum.

λ Library Screening. λ clones with overlapping insert DNAs covering SPI2 were obtained by standard methods (15) from a λ1059 library (16) containing inserts from a partial *Sau*3A digest of *S. typhimurium* LT2 genomic DNA. The library was

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Abbreviations: SPI, *Salmonella* pathogenicity island; ORF, open reading frame.

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obtained via K. Sanderson, from the Salmonella Genetic Stock Centre (SGSC), Calgary, Canada.

Mu*d*-P22 Lysogens. Radiolabeled DNA probes were hybridized to Hybond-N (Amersham) filters bearing DNA prepared from lysates of a set of *S. typhimurium* strains harboring Mu*d*-P22 prophages at known positions in the *S. typhimurium* genome. Preparation of mitomycin-induced Mu*d*-P22 lysates was as described (14, 17). The set of Mu*d*-P22 prophages was originally assembled by Benson and Goldman (18) and was obtained from B. Raupach (Stanford University School of Medicine, Stanford, CA).

Gel Electrophoresis and Southern Blot Hybridization. Gel electrophoresis was performed in 1% or 0.6% agarose gels in 0.5 × TBE. Gel-fractionated DNA was transferred to Hybond-N or -N+ membranes (Amersham) and stringent hybridization and washing procedures (permitting hybridization between nucleotide sequences with 10% or less mismatches) were as described by Holden *et al.* (19). For nonstringent conditions (permitting hybridization between sequences with 50% mismatches), filters were hybridized overnight at 42°C in 10% (vol/vol) formamide/0.25 M Na_2HPO_4/7% (wt/vol) SDS, and the most stringent step was with 20 mM Na_2HPO_4/1% SDS at 42°C. DNA fragments used as probes were labeled with [^{32}P]dCTP by using the Radprime system (GIBCO/BRL) or with [digoxigenin-11]dUTP and detected using the digoxigenin system (Boehringer Mannheim) according to the manufacturers' instructions, except that hybridization was performed in the same solution as that used for radioactively labeled probes. Genomic DNA was prepared for Southern blot hybridization as described (15).

Molecular Cloning and Nucleotide Sequencing. Restriction endonucleases and T4 DNA ligase were obtained from GIBCO/BRL. General molecular biology techniques were as described in Sambrook *et al.* (20). Nucleotide sequencing was performed by the dideoxy-nucleotide chain-termination method (21) using a T7 sequencing kit (Pharmacia). Sequences were assembled with the MACVECTOR 3.5 software or ASSEMBLYLIGN packages. Nucleotide and derived amino acid sequences were compared with those in the European Molecular Biology Laboratory (EMBL) and SwissProt databases by using the BLAST and FASTA programs of the GCG package from the University of Wisconsin (version 8) (22) on the network service at the Human Genome Mapping Project Resource Centre, Hinxton, U.K.

Virulence Tests. Groups of five female BALB/c mice (20–25 g) were inoculated p.o. or i.p. with 1:10 dilutions of bacteria suspended in physiological saline. For preparation of the inoculum, bacteria were grown overnight at 37°C in LB broth with shaking (50 rpm) and then used to inoculate fresh medium for various lengths of time until an OD_{560} of 0.4–0.6 unit had been reached. For cell densities of 5×10^8 colony-forming units/ml and above, cultures were concentrated by centrifugation and resuspended in saline. The concentration of these bacteria was checked by plating a dilution series of the inoculum onto LB agar plates. Mice were inoculated i.p. with 0.2 ml and p.o. by gavage with the same volume of inoculum. The LD_{50} values were calculated after 28 days by the method of Reed and Muench (23).

RESULTS

Localization of Transposon Insertions. The generation of a bank of *Salmonella typhimurium* mini-Tn*5* transposon mutants and the screen used to identify 43 mutants with attenuated virulence have been described (5). Transposons and flanking DNA regions were cloned from exconjugants by selection for kanamycin resistance or by inverse PCR. Nucleotide sequences of 300–600 bp of DNA flanking the transposons were obtained for 33 mutants. Comparison of these sequences with those in the DNA and protein databases indicated that 14 mutants resulted from transposon insertions into previously known virulence genes, 7 arose from insertions into new genes with similarity to known genes of the enterobacteria, and 12 resulted from insertions into sequences without similarity to entries in the DNA and protein databases (ref. 5 and this study).

Three lines of evidence suggested that 16 of 19 transposon insertions into new sequences were clustered in three regions of the genome, initially designated A, B, and C. (*i*) Comparing nucleotide sequences from regions flanking transposon insertion points with each other and with those in the databases showed that some sequences overlapped with one another or had strong similarity to different regions of the same gene. (*ii*) Southern blot analysis of genomic DNA digested with several restriction enzymes and probed with restriction fragments flanking transposon insertion points indicated that some transposon insertions were located on the same restriction fragments. (*iii*) When the same DNA probes were hybridized to plaques from a *S. typhimurium* λ DNA library, the probes from mutants that the previous two steps had suggested might be linked were found to hybridize to the same λ DNA clones. Thus two mutants (P9B7 and P12F5) were assigned to cluster A, five mutants (P2D6, P9B6, P11C3, P11D10, and P11H11) were assigned to cluster B, and nine mutants (P3F4, P4F8, P7A3, P7B8, P7G2, P8G12, P9G4, P10E11, and P11B9) were assigned to cluster C (see Fig. 2).

Hybridization of DNA probes from these three clusters to lysates from a set of *S. typhimurium* strains harboring locked-in Mu*d*-P22 prophages (17, 18) showed that the three loci were

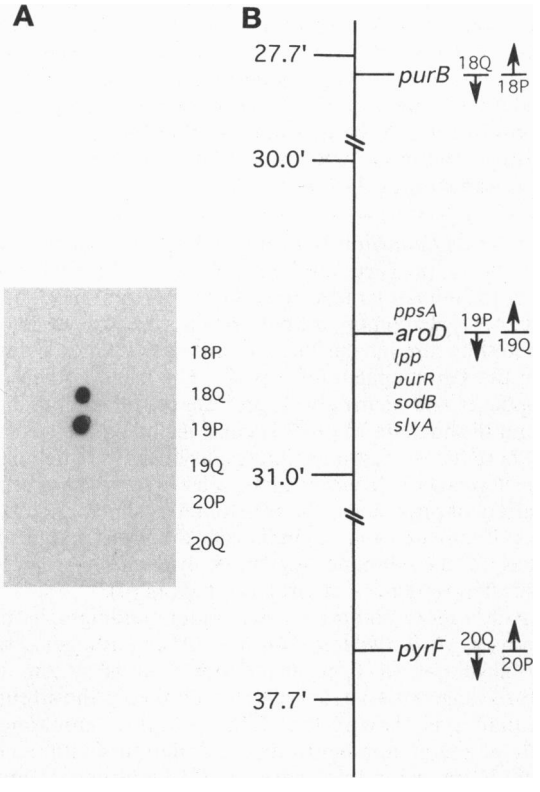

FIG. 1. Mapping of SPI2 on the *S. typhimurium* chromosome. (*A*) DNA probes from three regions of SPI2 were used in Southern blot hybridization analysis of lysates from a set of *S. typhimurium* strains harboring locked-in Mu*d*-P22 prophages. Lysates that hybridized to a 7.5-kb *Pst* I fragment (Fig. 2, probe A) are shown. The other two probes used hybridized to the same lysates (data not shown). (*B*) The insertion points and packaging directions of the phage are shown along with the map position in minutes (edition VIII, ref. 22). The phage designations correspond to the following strains: 18P, TT15242; 18Q, TT15241; 19P, TT15244; 19Q, TT15243; 20P, TT15246; 20Q, TT15245 (18). The locations of mapped genes are shown by horizontal bars and the approximate locations of other genes are indicated.

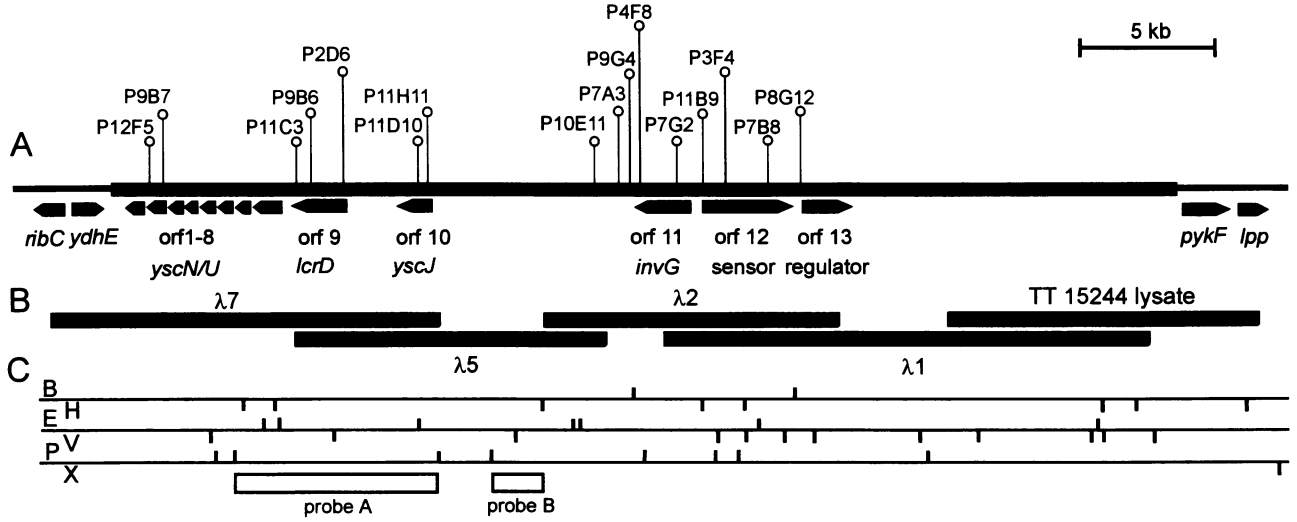

FIG. 2. Physical and genetic map of SPI2. (*A*) The positions of 16 transposon insertions are shown above the line. The extent of SPI2 is indicated by the thicker line. The position and direction of transcription of ORFs described in the text are shown by arrows below the line, together with the names of similar genes, with the exception of ORF12 and ORF13, whose products are similar to the sensor and regulatory components, respectively, of a variety of two component regulatory systems. (*B*) The location of overlapping λ clones and an *Eco*RI/*Xba* I restriction fragment from DNA of a lysate of a *S. typhimurium* strain TT15244 harboring a locked-in Mu*d*-P22 prophage are shown as solid bars. Only the portions of the λ clones that have been mapped are shown and the clones may extend beyond these limits. (*C*) The positions of restriction sites are marked (B, *Bam*HI; E, *Eco*RI; V, *Eco*RV; H, *Hin*dIII; P, *Pst* I; X, *Xba* I). The positions of the 7.5-kb *Pst* I fragment (probe A) used as a probe in Fig. 1 and that of the 2.2-kb *Pst* I–*Hin*dIII fragment (probe B) used as a probe in Fig. 4 are shown below the restriction map.

all located in the region from minutes 30 to 31 (edition VIII, ref. 11) (Fig. 1), indicating that the three loci were closely linked or constituted one large virulence locus. To determine whether any of the λ clones covering clusters A, B, and C contained overlapping DNA inserts, DNA fragments from the terminal regions of each clone were used as probes in Southern blot hybridization analysis of the other λ clones. Hybridizing DNA fragments showed that several λ clones overlap and that clusters A, B, and C formed one contiguous region (Fig. 2). DNA fragments from the ends of this region were then used to probe the λ library to identify further clones containing inserts representing the adjacent regions. No λ clones were identified that covered the extreme right terminus of the locus so this region was obtained by cloning a 6.5-kb *Eco*RI–*Xba* I fragment from a lysate of the Mu*d*-P22 prophage strain TT15244 (18).

Restriction mapping and Southern blot hybridization analysis were then used to construct a physical map of this locus (Fig. 2). To distinguish this locus from the well-characterized pathogenicity island containing the *inv/spa* gene cluster at minute 63 (edition VIII, ref. 11) (8, 9, 12, 24–27), we refer to the latter as SPI1 and have termed the new virulence locus SPI2.

Mapping the Boundaries of SPI2 on the *S. typhimurium* Chromosome. Nucleotide sequencing of λ clone 7 at the left side of SPI2 revealed the presence of an open reading frame (ORF) whose deduced amino acid sequence is ≥90% identical to the derived product of a segment of the *ydhE*‡ gene of *E. coli* and sequencing of the 6.5-kb *Eco*RI–*Xba* I cloned fragment on the right side of SPI2 revealed the presence of an ORF whose predicted amino acid sequence is ≥90% identical to pyruvate kinase I of *E. coli* encoded by the *pykF* gene (28). On the *E. coli* chromosome, *ydhE* and *pykF* are located close to one another, at minute 37–38 (29). Eleven nonoverlapping DNA fragments distributed along the length of SPI2 were used as probes in nonstringent Southern blot hybridization analysis of *E. coli* and *S. typhimurium* genomic DNA. Hybridizing DNA fragments showed that a region of approximately 40 kb containing SPI2 was absent from the *E. coli* genome and localized the boundaries of SPI2 to within 1 kb (Fig. 3). Comparison of the location of the *Xba* I site close to the right end of SPI2 (Fig. 2) with a map of known *Xba* I sites (30) at the minute 30 region of the chromosome (11) enables a map position of 30.7 minutes to be deduced for SPI2.

Structure of SPI2. Nucleotide sequencing of portions of SPI2 has revealed the presence of 13 ORFs (Fig. 2). The G+C content of approximately 26 kb of nucleotide sequence within SPI2 is 44.6%, compared to 47% for SPI1 (9) and 51–53% estimated for the entire *Salmonella* genome (31).

The complete deduced amino acid sequences of ORF1–11 are similar to those of proteins of type III secretion systems (6, 7), which are known to be required for the export of virulence determinants in a variety of bacterial pathogens of plants and animals (7). The predicted proteins of ORF1–8 (Fig. 2) are similar in organization and sequence to the products of the *yscN–U* genes of *Yersinia pseudotuberculosis* (32), to *invC/spaS* of the *inv/spa* cluster in SPI1 of *S. typhimurium* (8, 9), and to *spa47/spa40* of the *spa/mxi* cluster of *Shigella flexneri* (33–36). For example, the predicted amino acid sequence of ORF3 (Fig. 2) is 50% identical to YscS of *Y. pseudotuberculosis* (32), 34% identical to Spa9 from *Shigella flexneri* (36), and 37% identical to SpaQ of SPI1 of *S. typhimurium* (9). The predicted protein product of ORF9 is closely related to the LcrD family of proteins with 43% identity to LcrD of *Yersinia enterocolitica* (37), 39% identity to MxiA of *Shigella flexneri* (33), and 40% identity to InvA of SPI1 (24). Partial nucleotide sequences for the remaining ORFs shown in Fig. 2 indicate that the predicted protein from ORF10 is most similar to *Y. enterocolitica* YscJ (38), a lipoprotein located in the bacterial outer membrane, with ORF11 similar to *S. typhimurium* InvG, a member of the PulD family of translocases (39). ORF12 and ORF13 show significant similarity to the sensor and regulatory subunits, respectively, from a variety of proteins making up two-component regulatory systems (40). There is ample coding capacity for further genes between ORF9 and ORF10, between ORF10 and ORF11, and between ORF13 and the right end of SPI2.

SPI2 Is Conserved Among and Is Specific to the *Salmonellae*. A 2.2-kb *Pst* I–*Hin*dIII fragment located at the centre of SPI2 (Fig. 2, probe B) lacking sequence similarity to entries in the DNA and protein databases was used as a probe in Southern blot hybridization analysis of genomic DNA from *Salmonella*

‡Eberhardt, S.M.R., Richter, G., Gimbel, W., Werner, T. & Bacher, A. (1994) SwissProt accession number P37340.

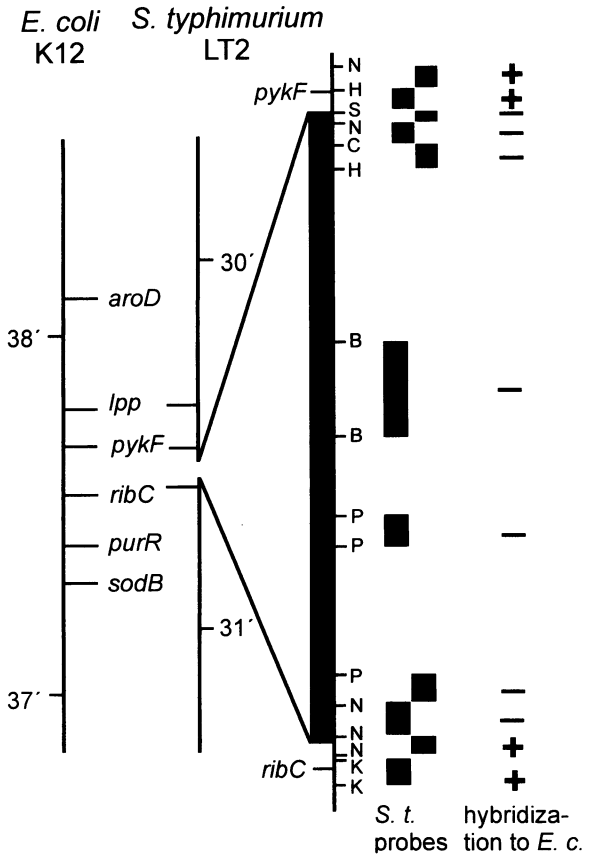

FIG. 3. Mapping the boundaries of SPI2. (*A*) The positions of mapped genes at minutes 37–38 on the *E. coli* K-12 chromosome are aligned with the corresponding region of the *S. typhimurium* LT2 chromosome (minutes 30–31). An expanded map of the SPI2 region is shown with 11 *S. typhimurium* (S. t.) DNA fragments used as probes (thick bars) and the restriction sites used to generate them (B, *Bam*HI; C, *Cla* I; H, *Hin*dII; K, *Kpn* I; P, *Pst* I; N, *Nsi* I; S, *Sal* I). Probes that hybridized to *E. coli* K-12 (E. c.) genomic DNA are indicated by +; those that failed to hybridize are indicated by −.

serovars and other pathogenic bacteria (Fig. 4*A*). DNA fragments hybridizing under nonstringent conditions showed that SPI2 is present in *S. aberdeen, S. gallinarum, S. cubana*, and *S. typhi* and is absent from EPEC, EHEC, *Y. pestis, Shigella flexneri, V. cholera*, and *Staphylococcus aureus*. Thus SPI2 is conserved among and is likely to be specific to the *Salmonellae*. To determine whether the organization of the locus is conserved among the *Salmonella* serovars tested, stringent Southern blot hybridizations with genomic DNA digested with two further restriction enzymes were carried out. Hybridizing DNA fragments showed that there is some heterogeneity in the arrangement of restriction sites between *S. typhimurium* LT2 and *S. gallinarum, S. cubana*, and *S. typhi* (Fig. 4*B*). Furthermore, *S. gallinarum* and *S. typhi* contain additional hybridizing fragments to those present in the other *Salmonellae* examined, suggesting that regions of SPI2 have been duplicated in these species.

SPI2 Is Required for Virulence in Mice. Previous experiments showed that the LD_{50} values for i.p. inoculation of transposon mutants P3F4, P7G2, P9B7, and P11C3 were at least 100-fold greater than the wild-type strain (5). To clarify the importance of SPI2 in the process of infection, the p.o. and i.p. LD_{50} values for mutants P3F4 and P9B7 were determined (Table 1). Both mutants showed a reduction in virulence of at least five orders of magnitude by either route of inoculation in comparison with the parental strain. This profound attenuation of virulence by both routes of inoculation demonstrates that SPI2 is required for events in the infective process after epithelial cell penetration in BALB/c mice.

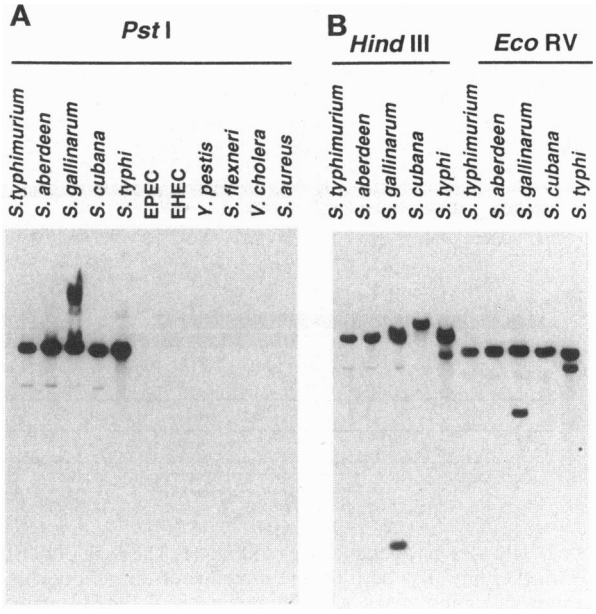

FIG. 4. SPI2 is conserved among and specific to the *Salmonellae*. Genomic DNA from *Salmonella* serovars and other pathogenic bacteria was digested with *Pst* I (*A*) or with *Hin*dIII or *Eco*RV (*B*) and subjected to Southern blot hybridization analysis using a 2.2-kb *Pst* I–*Hin*dIII fragment from λ clone 7 as a probe (Fig. 2, probe B). The filters were hybridized and washed under nonstringent (*A*) or stringent (*B*) conditions.

DISCUSSION

We have identified a hitherto unknown virulence locus in *S. typhimurium* of approximately 40 kb located at minute 30.7 on the chromosome by mapping the insertion points of a group of signature-tagged transposon mutants with attenuated virulence (5). We refer to this locus as SPI2 to distinguish it from the SPI containing the *inv/spa* virulence genes at 63 minutes (edition VIII, ref. 11), which we suggest be renamed SPI1. SPI1 and SPI2 both encode components of type III secretion systems. However, these secretion systems are functionally distinct.

Of 19 mutants that arose from insertions into new genes (ref. 5 and this study), 16 mapped to the same region of the chromosome. It is possible that mini-Tn*5* insertion occurs preferentially in SPI2. Alternatively, as the negative selection used to identify mutants with attenuated virulence (5) was very stringent (reflected by the high LD_{50} values for SPI2 mutants), it is possible that, among the previously unknown genes, only mutations in those of SPI2 result in a degree of attenuation sufficient to be recovered in the screen. The failure of previous searches for *S. typhimurium* virulence determinants to identify SPI2 might stem from reliance on cell culture assays rather than a live animal model of infection. A previous study that identified regions of the *S. typhimurium* LT2 chromosome unique to *Salmonellae* (41) located one such region (RF333) to minutes 30.5–32. Therefore, RF333 may correspond to SPI2.

Comparisons with the type III secretion systems encoded by the virulence plasmids of *Yersinia* and *Shigella*, as well as with SPI1 of *Salmonella*, indicate that SPI2 encodes the basic

Table 1. LD_{50} values of *S. typhimurium* strains

Strain	LD_{50}, cfu	
	i.p.	p.o.
12023 (wild type)	4.2	6.2×10^4
P3F4	1.5×10^6	$>5 \times 10^9$
P9B7	$>1.5 \times 10^6$	$>5 \times 10^9$

cfu, Colony-forming unit(s).

structural components of the secretory apparatus. Furthermore, the order of ORF1–8 in SPI2 is the same as the gene order in homologs in *Yersinia* and *Shigella*, and SPI1 of *S. typhimurium*. The organization and predicted amino acid sequence of genes within SPI2 are no more closely related to those in SPI1 than to the corresponding proteins of *Yersinia*. This, together with the low G+C content of SPI2, suggests that SPI2, like SPI1 (41–43), was acquired independently by *S. typhimurium* via horizontal transmission. The proteins encoded by ORF12 and ORF13 show strong similarity to bacterial two-component regulators (40) and could regulate either ORF1–11 and/or the secreted proteins of this system.

Mills *et al.* (12) have proposed that the chromosomal region containing the *inv/spa* gene cluster is a pathogenicity island (PAI). PAIs are large defined regions of DNA that contain virulence genes and have been found in a variety of pathogenic bacteria, including *Yersinia* (44), *Listeria monocytogenes* (45), uropathogenic *E. coli* (46, 47), and enteropathogenic *E. coli* (48). However, unlike the PAIs found in *Yersinia* and uropathogenic *E. coli*, which are unstable and lost at high frequency (44, 49), both SPI1 and SPI2 appear to be stable in *S. typhimurium*.

Many genes in SPI1 have been shown to be important for entry of *S. typhimurium* into epithelial cells. This process requires bacterial contact (2) and results in cytoskeletal rearrangements leading to localized membrane ruffling (50, 51). The role of SPI1 and its restriction to this stage of the infection is reflected in the approximately 50-fold attenuation of virulence in BALB/c mice inoculated p.o. with SPI1 mutants and by the fact that SPI1 mutants show no loss of virulence when administered i.p. (8). The second observation also explains why no SPI1 mutants were obtained in our screen (5). In contrast, mutants in SPI2 are profoundly attenuated after both p.o. and i.p. inoculation. This shows that, unlike SPI1, SPI2 is required for virulence in mice after epithelial cell penetration, but these findings do not exclude a role for SPI1 in this early stage of infection. Further characterization of SPI2 may help to identify the putative target(s) of this secretion system and the nature of their interaction with the host.

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