The *shdA* Gene Is Restricted to Serotypes of *Salmonella enterica* Subspecies I and Contributes to Efficient and Prolonged Fecal Shedding

ROBERT A. KINGSLEY, KARIN VAN AMSTERDAM, NAOMI KRAMER, AND ANDREAS J. BÄUMLER*

Department of Medical Microbiology and Immunology, College of Medicine, Texas A&M University Health Science Center, College Station, Texas 77843-1114

Received 12 November 1999/Returned for modification 4 January 2000/Accepted 10 February 2000

Little is known about factors which enable *Salmonella* serotypes to circulate within populations of livestock and domestic fowl. We have identified a DNA region which is present in *Salmonella* serotypes commonly isolated from livestock and domestic fowl (*S. enterica* subspecies I) but absent from reptile-associated *Salmonella* serotypes (*S. bongori* and *S. enterica* subspecies II to VII). This DNA region was cloned from *Salmonella* serotype Typhimurium and sequence analysis revealed the presence of a 6,105-bp open reading frame, designated *shdA*, whose product's deduced amino acid sequence displayed homology to that of AIDA-I from diarrheagenic *Escherichia coli*, MisL of serotype Typhimurium, and IcsA of *Shigella flexneri*. The *shdA* gene was located adjacent to *xseA* at 52 min, in a 30-kb DNA region which is not present in *Escherichia coli* K-12. A serotype Typhimurium *shdA* mutant was shed with the feces in reduced numbers and for a shorter period of time compared to its isogenic parent. A possible role for the *shdA* gene during the expansion in host range of *S. enterica* subspecies I to include warm-blooded vertebrates is discussed.

*Salmonella* serotypes are a frequent constituent of the intestinal flora of poikilothermic animals. The percentage of apparently healthy, cold-blooded vertebrates which harbor *Salmonella* serotypes ranges from 74 to 94% (20, 28, 32, 34, 59), and these bacteria could thus be considered part of the normal intestinal flora (23, 48). *Salmonella* serotypes are also commonly isolated from a fraction (usually <20%) of warm-blooded animal hosts (15, 31, 49, 54). Although chronic carriers, which appear healthy, are observed within the human population and among warm-blooded animals (22, 27, 35, 40), *Salmonella* serotypes are commonly associated with infection in these hosts (55). Consequently *Salmonella* serotypes are regarded as pathogens rather than part of the normal intestinal flora of homeothermic animals.

On the basis of multilocus enzyme electrophoresis and comparative sequence analysis of orthologous genes, two species, *S. enterica* and *S. bongori*, have been assigned to the genus *Salmonella* (18, 46). *S. enterica* is further subdivided into seven subspecies designated with roman numerals (18, 44). While *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, VI, and VII are mainly associated with cold-blooded vertebrates, members of *S. enterica* subspecies I are frequently isolated from avian and mammalian hosts (44). For instance, of the 90,201 *Salmonella* isolates collected between 1977 and 1992 by the German National Reference Center for Enteric Pathogens from humans and warm-blooded animals, 89,798 isolates (99.55%) belonged to *S. enterica* subspecies I (1). Currently it is not clear which virulence mechanisms are responsible for the apparent adaptation of *S. enterica* subspecies I to circulation within populations of warm-blooded animals.

*S. bongori* or *S. enterica* subspecies II to VII are able to infect humans, colonize the intestine and cause disease (1). Human infections with serotypes of *S. bongori* and *S. enterica* subspecies II to VII are rare and are usually the result of contact with reptiles (21, 29, 42, 60). The symptoms of intestinal and extraintestinal infections caused by reptile-associated *Salmonella* serotypes in humans are, however, indistinguishable from those produced by nontyphoidal serotypes of *S. enterica* subspecies I (1). The rate at which serotypes of *Salmonella* enter into contact with their end host appears to be determined by the organism's ability to colonize the intestine and spread within a host population. Among cold-blooded vertebrates, which are usually infected only once and have a rapid turnover of infection rates, this can be achieved by increasing the rate of contact between the organism and the host to such an extent that, on average, more than one secondary case of infection from a primary case is achieved. The average number of animals in a susceptible host population which become infected from a single case can be defined as the basic case reproductive number, *R*₀ (3). The basic case reproductive number of *S. enterica* subspecies I serotypes for higher vertebrates must therefore be greater than one, since these pathogens circulate in warm-blooded host populations. The absence of *S. bongori* and *S. enterica* subspecies II to VII serotypes from populations of livestock or domestic fowl, on the other hand, suggests that their basic case reproductive number for higher vertebrates is less than one, a property apparently independent of their ability to cause illness in these hosts (1). Thus, an expansion in host range may involve the acquisition of one or more genetic determinants by a common ancestor of *S. enterica* subspecies I which increased the basic case reproductive number (but not necessarily the lethality) of this organism for warm-blooded vertebrates. To predict how acquisition of new genetic material by a common ancestor of the *S. enterica* subspecies I lineage may have contributed to its expansion in host range, it is helpful to apply theoretical models which
combine epidemiology with population biology (3). In the case of direct transmission (by the fecal-oral route or any other route), the basic case reproductive number of a pathogen is directly proportional to the duration, D, for which an infected host can transmit the disease; the probability, b, at which the disease is transmitted from an infected animal to a susceptible host; and the density of susceptible hosts, X (2):

\[ R_0 = \beta DX \]  

(1)

An infected host can transmit the disease until it either dies of natural causes (at the natural mortality rate, \( h \)), is killed by the pathogen (at the disease-induced mortality rate, \( \alpha \)), or is able to clear the infection (at the clearance rate, \( v \)) (3). Thus the average lifespan of an infectious host, \( D \), can be described as follows:

\[ D = \frac{1}{\alpha + b + v} \]  

(2)

After combining equations 1 and 2 it becomes clear that a reduction of either the clearance rate, \( v \), or the disease induced mortality rate, \( \alpha \), will result in an increase in the basic case reproductive number, \( R_0 \), of a pathogen:

\[ R_0 = \frac{\beta DX}{\alpha + b + v} \]  

(3)

There is no evidence that members of S. enterica subspecies I are less virulent or cause lower mortality rates in warm-blooded hosts than serotypes of S. bongori or S. enterica II to VII. However, it is possible that a common ancestor of the S. enterica subspecies I lineage may have increased its basic case reproductive number for warm-blooded animals by reducing the rate at which the infection is cleared from the feces. The genetic determinants responsible for this phenotype are expected to be present in S. enterica subspecies I but absent from serotypes of S. bongori and S. enterica subspecies II to VII.

Here we describe the identification of a gene, termed shdA, which is specific to S. enterica subspecies I serotypes and study its role in fecal shedding during S. enterica serotype Typhimurium infection of mice. This analysis is relevant for human health, since little is known about the genetic determinants required for circulation of enteric pathogens within animal reservoirs from which we draw our food supply.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Strains CL1509 (aroA\(\rightarrow\)Tn10), IR715 (virulent nalidixic acid resistant derivative), AJB82 (aroA\(\rightarrow\)Tn10 mcrA::TphoA4) and AJB75 (IR715 mcrA::TphoA) are derivatives of serotype Typhimurium strain ATCC 14028 (10, 56, 57). The Salmonella reference B and SARC collections have been published recently (17, 18). Escherichia coli strains S17-1 kpr and DH5\(\alpha\) are described elsewhere (26, 53). Strains were cultured aerobically at 37°C in Luria-Bertani (LB) broth supplemented with the following antibiotics as appropriate at the indicated concentrations: carbenicillin, 100 mg/liter (LB +Cb); chloramphenicol, 30 mg/liter (LB + Cm); tetracycline, 20 mg/liter (LB + Tc); kanamycin 60 mg/liter (LB + Km); or nalidixic acid, 50 mg/liter (LB + NaH). For single infection experiments, groups of 12 BALB/c mice were inoculated with 10\(^9\) CFU of either CL1509 (aroA\(\rightarrow\)Tn10::Tet) or RAK7 (aroA\(\rightarrow\)Tn10::Tet shdA::Cm\(^\prime\)). The presence of inoculum strain in fecal pellets was determined on 29 days postinfection (days 1 to 16, 18, 21, 24, 27, 31, 34, 37, 39 for postinfection; 72, and 79 for preinfection). Appropriate dilutions of bacterial cultures in a 0.2-ml volume. Lethal morbidity was recorded at 28 days postinfection, and the estimated LD\(_{50}\) was calculated by the method of Reed and Muench (45).

**RESULTS**

**Identification of a DNA region restricted to S. enterica subspecies I.** We have recently screened a bank of 400 S. enterica serotype Gallinarum Mud-Cam transposon mutants for virulence in day-of-hatch White Leghorn chicks. Virulence data obtained for individual mutants during the initial screen were inconsistent with experiments performed using this animal model to confirm attenuation of individual mutants. We determined the inconsistency of data to be the result of the antibiotic history from battery-reared chicks. The fact that virulence defects could not be confirmed for mutants identified by the dideoxy chain termination method (50), using an AutoRead Sequencing Kit (Pharmacia) and an ALF automatic sequenator. The nucleotide sequences were analyzed using the MacVector 6.0.1 software package (Oxford Molecular Group).

**Construction of mutants.** Bacteriophage P22 HII/105/1 int\(^\prime\) was used for generalization of the transduction of the aroA\(\rightarrow\)Tn10 marker from serotype Typhimurium strain CL1509 into RAK8 or AJB75 (10). Transductants were routinely purified from contaminating phage by streaking the strain twice for single colonies on Evans blue uridine plates (16). Subsequently, strains were tested in a cross test for P22 sensitivity. Transductants were tested for growth on M9 minimal medium agar plates and on minimal medium agar plates supplemented with aromatic amino acids (39). For construction of a shdA mutant, a 3-kb fragment of cosmid pRK624 was cloned into pBluecript KS\(^\prime\) (52) to give rise to plasmid pRA38. A chloramphenicol acetyltransferase (cat) gene was ligated into the BamHI site of the shdA open reading frame cloned in pRA39 (see Fig. 3). The insert of the resulting plasmid (pRA55) was excised with EcoRI and SalI and cloned into the EcoRI- and SalI-restricted suicide vector pGPT704 (33) to give rise to plasmid pRA56. Exconjugants of a mating between serotype Typhimurium strain IR715 and col col strain S17-1 (pRA56) were used to construct plasmids. An exconjugant which was resistant to chloramphenicol (shdA::cat allele) but sensitive to carbencillin (through loss of pGPT704) was identified by patching individual colonies on LB + Cb plates and was termed RAK1.

**Animal experiments.** Six- to eight-week-old female BALB/c (ByJ; Jackson Laboratory) mice were used in this study. Bacteria were routinely cultured as standing overnight cultures prior to infection. In all experiments the bacterial titer of the inoculum was determined by spreading serial 10-fold dilutions on agar plates containing appropriate antibiotics and determining the number of CFU.

The intestinal organ culture model has been described previously (7). The intestine was ligated at the distal end, filled with 1 ml of a bacterial suspension consisting of a mixture of the two mutant strains, and then ligated at the proximal end and incubated for 30 min at 37°C in 5% CO\(_2\). Nonadherent bacteria were removed by five washes in phosphate-buffered saline (PBS), and sections of intestinal wall were homogenized in 5 ml of PBS. Dilutions were spread on LB plates containing the appropriate antibiotics. Experiments were repeated with organs from three different animals.

For competitive infection experiments, groups of four mice were inoculated by oral gavage with an approximately 1:1 mixture of mutant and isogenic parent strains (10\(^7\) CFU of approximative 1\(^\) CFU/mouse. Fecal pellets were collected daily and homogenized in 1 ml of PBS. The limit of detection was approximately 0.08 CFU/mg of feces. Dilutions of fecal pellets were plated on LB plates containing the appropriate antibiotics. Data were normalized by dividing the output ratio (CFU of mutant/CFU of wild type) by the input ratio (CFU of mutant/CFU of wild type). In case only one bacterial strain was recovered from fecal pellets, the limit of detection was determined for the missing strain and used to calculate of a minimum mutant/wild type ratio. All data were converted logarithmically prior to the calculation of averages and statistical analysis. Student’s t test was used to determine whether the mutant/wild type ratio in specimens recovered from infected organs or fecal pellets was significantly different from the mutant/wild type ratio present in the inoculum.

For single infection experiments, groups of 12 BALB/c mice were inoculated with 10\(^9\) CFU of either CL1509 (aroA\(\rightarrow\)Tn10::Tet) or RAK7 (aroA\(\rightarrow\)Tn10::Tet shdA::Cm\(^\prime\)). The presence of inoculum strain in fecal pellets was determined on 10 days by the 29 days period of postinfection (days 1 to 16, 18, 21, 24, 27, 31, 34, 37, 39 for postinfection; 72, and 79 for preinfection). Appropriate dilutions of bacterial cultures in a 0.2-ml volume. Lethal morbidity was recorded at 28 days postinfection, and the estimated LD\(_{50}\) was calculated by the method of Reed and Muench (45).

The 50% lethal morbidity dose (LD\(_{50}\)) of serotype Typhimurium mutants was estimated by infecting groups of four mice intragastrically with serial 10-fold dilutions of bacterial cultures in a 0.2-ml volume. Lethal morbidity was recorded at 28 days postinfection, and the estimated LD\(_{50}\) was calculated by the method of Reed and Muench (45).

The 50% lethal morbidity dose (LD\(_{50}\)) of serotype Typhimurium mutants was estimated by infecting groups of four mice intragastrically with serial 10-fold dilutions of bacterial cultures in a 0.2-ml volume. Lethal morbidity was recorded at 28 days postinfection, and the estimated LD\(_{50}\) was calculated by the method of Reed and Muench (45).
in this screen prompted us to discontinue the study. However, prior to this, the DNA flanking the Mud-Cam insertion was cloned from one mutant, labeled, and used as a probe to determine its phylogenetic distribution. Southern blot analysis was performed with genomic DNA prepared from SARC (18). This collection consists of 16 Salmonella serotypes representing S. bongori (formerly S. enterica subspecies V) and S. enterica subspecies I to VII. The 500-bp p5A8 DNA probe, derived from mutant G5A8, hybridized with genomic DNA of strains from subspecies I, but no hybridization signal was obtained with genomic DNA from isolates of S. bongori or S. enterica subspecies II to VII. Since the host range of S. enterica subspecies I differs from that of S. bongori or S. enterica subspecies II to VII we decided to further characterize this DNA region.

A cosmid (pRK824) containing the S. enterica subspecies I specific DNA region was cloned from an S. enterica serotype Typhimurium bank (38) by hybridization with probe p5A8. Restriction analysis of cosmid pRK824 indicated that it carried an insert of approximately 28 kb. To confirm that the cloned DNA region was restricted to S. enterica subspecies I, a 3-kb ClaI restriction fragment hybridizing with probe p5A8 was cloned to give rise to plasmid pRA38. The DNA probe derived from plasmid pRA38 hybridized with genomic DNA from S. enterica subspecies I but not genomic DNA from S. bongori or S. enterica subspecies II to VII (Fig. 1). To determine the distribution of the pRA38 DNA probe within S. enterica subspecies I, we used the SARB collection, which includes 72 strains representing 37 serotypes of S. enterica subspecies I (17). A DNA probe derived from plasmid pRA38 hybridized with 69 of the 72 strains of SARB collection. Southern blot analysis of 21 representative strains from SARB collection is shown in Fig. 1. No hybridization signal was obtained with genomic DNA from strains D1t (S. enterica serotype Decatur), Ts1 (S. enterica serotype Typhuisus) and En2 (S. enterica serotype Enteritidis). The multilocus enzyme electrophoresis profile of strain En2 is only distantly related to that of other S. enterica serotype Enteritidis clones (En1, En3, and En7) which are present in SARB and do hybridize with pRA38 (17).

The shdA gene is located in the xseA-hisS intergenic region. The cosmid (pRK824) contained at least one border of the subspecies I specific DNA region, since a DNA probe (pRA58) generated from a 1.6-kb ClaI restriction fragment hybridized with all serotypes of the SARC and SARB collections (Fig. 1). Nucleotide sequence analysis of pRA58 revealed that it contained the 3' end of the serotype Typhimurium xseA gene, which encodes the large subunit of exonuclease VII. Since this gene is shared by both E. coli and serotype Typhimurium, it is likely to account for the hybridization signal observed with the S. bongori or S. enterica subspecies II to VII serotypes. Sequence homology between the E. coli and serotype Typhimurium sequence ended 21 bp prior to the 3' end of xseA, which defined the left end of the S. enterica subspecies I specific DNA region. In the sequence of the E. coli K-12 genome, the xseA gene is located between the guaAB and hisS loci at 54 min (14). However, genetic mapping data suggest that in serotype Typhimurium, guaAB and hisS are separated by a 30-kb DNA region which is absent from E. coli (Fig. 2) (47). Our data are consistent with the presence of the shdA gene on a genetic island present in the xseA-hisS intergenic region, which may be as large as 30 kb.

The nucleotide sequence analysis was extended to include a total of 6,831 bp (GenBank accession no. AF091269). A single open reading frame of 6,105 bp transcribed in the opposite direction to xseA was identified and designated shdA. A putative termination loop was identified downstream of the translational stop codon of shdA (stem, bp 6482 to 6495; loop, bp 6496 to 6499; stem, bp 6500 to 6514). The C-terminal domain (477 amino acids) of the predicted ShdA protein exhibited homology to the C-terminal 440 amino acids of AIDA (34% identity) from diffusely adhering E. coli (11), the C-terminal 501 amino acids of MisL (36% identity) from serotype Typhimurium (13), and the C-terminal 353 amino acids of IcsA (VirG) (30% identity) from S. flexneri (12, 36). The C terminus also contained five copies of a 12-amino-acid repeat, which exhibited no homology to sequences in available databases (Fig. 3). Using the SignalP program (41a), a putative signal peptide was identified at the amino terminus of ShdA. Similar to AIDA, MisL, and IcsA, the signal peptide was atypical, with the predicted cleavage site (indicated by a slash) following the alanine residue at position 60 (LAMA/DNQV). The 6496 to 6499; stem, bp 6500 to 6514)

FIG. 1. Phylogenetic distribution of the shdA gene within the genus Salmonella. Southern blot analysis using representative serotypes of S. enterica (subspecies are indicated by roman numerals) and S. bongori is shown. Genomic DNA prepared from the serotypes indicated on the left (strain designations are indicated in parentheses) was hybridized with DNA probes pRA58 (left panel) and pRA38 (right panel). The location of these DNA probes (closed bars) relative to xseA and shdA (arrows) is indicated on the map shown at the top.
open reading frame was disrupted by insertion of a cat gene was constructed by allelic exchange and was designated RAK1. The insertion mutant was confirmed by Southern blot analysis of chromosomal DNA prepared from RAK1 and hybridization with the pRA38 DNA probe (data not shown). It is unlikely that insertional inactivation of shdA gene, since the xseA and xseA genes are transcribed in opposite orientations. The LD$_{50}$ of strain RAK1 (shdA aroA) was still shed with the feces of three mice. (E.c.) strain containing a mutation in shdA, has been described by Riley and Krawiec (47) and is shown as an open bar.

**FIG. 2.** (Top) Comparison of the nucleotide sequences from *E. coli* (E.c.) and *S. enterica* serotype Typhimurium (S.t.) at the left boundary of the island. A putative termination loop located downstream of the shdA gene is indicated by arrows. (Bottom) Comparison of the genetic maps of *E. coli* and *S. enterica* serotype Typhimurium flanking the xseA gene. An approximately 30-kb DNA loop in the guaB-hisS intergenic region, which is present in serotype Typhimurium but absent from *E. coli*, has been described by Riley and Krawiec (47) and is shown as an open bar.

**Effect of a mutation in shdA on fecal shedding.** We determined the contribution of shdA to bacterial shedding in the mouse typhoid model of serotype Typhimurium infection. Serotype Typhimurium causes lethal signs of disease in mice starting at day 5 postinfection. Thus, in order to study fecal shedding beyond day 5 postinfection, strain RAK1 (shdA) was attenuated for mouse virulence by introducing a mutation in *aroA*. Serotype Typhimurium *aroA* mutants are able to attach to and invade the intestinal mucosa and colonize deeper tissues but are unable to multiply rapidly at these sites. Since bacterial shedding results from bacterial colonization of an animal, we reasoned that inactivation of *aroA* was unlikely to mask the effect of other genes on shedding. To assess the effect of a mutation in shdA on bacterial shedding, a group of four mice was infected with equal numbers of CL1509 (aroA) and RAK7 (shdA aroA) bacteria, and the bacteria were recovered from fecal pellets on subsequent days. This analysis revealed that a mutation in shdA significantly decreased the number of serotype Typhimurium organisms shed in the feces ($P < 0.01$ at day 6 postinfection). The experiment was discontinued at day 6 postinfection, when RAK7 (shdA aroA) was not recovered from the fecal pellets of three mice, while strain CL1509 (aroA) was still shed with the feces of three animals.

The experiment was repeated with a group of six mice, and shedding was monitored until day 35 postinfection. All mice shed the inoculum on day 1 postinoculation, but on subsequent days shedding was intermittent and some animals cleared the inoculum. Again, CL1509 (aroA) was recovered in significantly higher numbers from fecal pellets than RAK7 (shdA aroA). More importantly, CL1509 was shed for a longer period than the shdA mutant (RAK7). Figure 4 shows the combined results of both shedding experiments. These data show that a mutation in shdA reduced the duration of bacterial shedding, which is indicative of an increased rate by which the host could clear serotype Typhimurium from intestinal contents shed with the feces. The shedding defect attributed to the shdA mutation was confirmed using single inoculation of groups of 12 mice with $10^9$ CFU of either RAK7 (*aroA* shdA) or the CL1509 (*aroA*) parental strain. Shedding in fecal pellets was scored for the presence or absence of *Salmonella* in each inoculum group on 29 occasions over a 79-day period postinoculation. On 14 occasions during this period, a greater number of mice inoculated with the parental strain (CL1509) were shedding serotype Typhimurium than were those inoculated with the *shdA* mutant (RAK7). The opposite was true on only three occasions. Statistical analysis of these data using the Wilcoxon signed-rank test indicated that the *shdA* mutant was cleared significantly earlier than the parental strain ($P < 0.01$). Overall, these data suggest that a mutation in *shdA* increased the clearance rate of serotype Typhimurium from murine feces.

**Effect of a mutation in invA on fecal shedding.** During competitive infection experiments, serotype Typhimurium strains carrying a mutation in *invA* are absent from feces more frequently than the wild type on days 3 and 5 postinoculation (10). However, the shedding defect previously reported for *invA* mutants was based on observation restricted to two occasions postinoculation. Observations from shedding experiments described above indicated that shedding is highly variable from day to day. In order to investigate whether a serotype Typhimurium strain containing a mutation in *invA* has a similar shedding defect to that observed for strains containing a mutation in *shdA*, eight mice were inoculated with an equal mixture of strain AJB82 (*invA* aroA) and its isogenic parental strain (CL1509). Fewer mice shed the *invA* mutant and at lower numbers at earlier time points. However, the opposite was observed at later time points (Fig. 5). Although a mutation in *invA* reduced bacterial shedding at early times postinfection, there was no evidence for an effect on bacterial clearance from the feces at the end of the experiment.

A mutation in *shdA* does not affect colonization of the villous intestine or Peyer’s patches in an intestinal organ culture model. It is known that *invA* is required for invasion of the mucosal epithelium, particularly at the Peyer’s patches (25). To compare the contributions of *shdA* and *invA* to colonization of the small intestine, we used the intestinal organ culture model (7). Equal numbers of RAK1 (shdA) and its parent (IR715) were injected into loops formed from fresh mouse ileum, and following a 30-min incubation period, CFU of each strain were enumerated in the villous intestine and Peyer’s patch regions. No significant difference in colonization of these tissues by the *shdA* strain and parental strain was observed. In a second experiment, an equal mixture of a serotype Typhimurium *invA* mutant (AJB75) and its isogenic parent (IR715) was inoculated into ligated intestinal loops. The *invA* mutant (AJB75)
was recovered in significantly lower numbers from Peyer's patches than the parental strain, thus confirming the role of SPI1 in colonizing this organ.

**DISCUSSION**

A primary pathogen can be defined as an organism which is capable of entering a host, finding a unique niche in which to multiply, avoiding or subverting the host defenses, and being transmitted to a susceptible host (24). All members of the genus *Salmonella* fit this description, as they are pathogenic for humans (1). However, serotypes of *S. enterica* subspecies I differ from *S. bongori* and *S. enterica* subspecies II to VII serotypes with regard to animal reservoir. While human infections with *S. bongori* and *S. enterica* subspecies II to VII are rare and result from contact with reptiles (21, 29, 42, 60),
serotypes of *S. enterica* subspecies I are frequently associated with disease, and most cases can be traced back to livestock or domestic fowl (4, 41). Thus, it could be speculated that serotypes of *S. enterica* subspecies I possess one or more genes which enable these pathogens to invade, persist, and spread within warm-blooded host populations, thereby resulting in their introduction into food items originating from domesticated animals. *S. bongori* and *S. enterica* subspecies II to VII, on the other hand, lack these genes and are unable to circulate in populations of livestock and domestic fowl.

We characterized *shdA*, a gene encoded on a genetic island which is present in serotypes of *S. enterica* subspecies I. Unlike previously identified virulence gene clusters, such as SPI1, SPI2, SPI3, *agf*, *fim*, *lpf*, and *spv*, the *shdA* gene was absent from lineages other than subspecies I (6, 13, 19, 30, 37). Although virulence determinants, which are restricted to subspecies I have been identified previously, these are present in only a small number of serotypes. For instance, the SARB collection which consists of 72 strains from *S. enterica* subspecies I, contains 3 isolates carrying the viaB region, 10 isolates possessing the *sef* operon, and 9 isolates hybridizing with the *pef* operon (6, 51). In contrast, *shdA* was present in 69 of the 72 strains of the SARB collection, suggesting that it was acquired early in the divergence of the *S. enterica* subspecies I lineage (Fig. 1). It has been postulated that the ability of *S. enterica* subspecies I serotypes to circulate in populations of warm-blooded animals is a new trait, since extant serotypes of all other phylogenetic lineages within the genus *Salmonella* are associated with cold-blooded vertebrates (8, 43). Our data suggest that this expansion in host range to include warm-blooded vertebrates was accompanied by acquisition of the *shdA* gene by a common ancestor of *S. enterica* subspecies I.

Our results show that mutational inactivation of *shdA* resulted in recovery of serotype Typhimurium at lower numbers and for a shorter period of time from murine fecal pellets than its isogenic parent (Fig. 4). It is unlikely that *shdA* is the only factor involved in prolonged fecal shedding of serotype Typhimurium from mice. Indeed, previous studies have shown that Typhimurium strains containing a mutation in *invA* are less likely to be recovered from fecal pellets of mice at days 3 and 5 postinfection than their isogenic parent (10). However, comparison of the shedding defects of strains AJ882 (*invA*) and RAK7 (*shdA*) demonstrated that inactivation of *shdA* reduced bacterial shedding at later time points (day 11 postinfection or subsequent days) to a greater extent than a mutation in *invA* (Fig. 4 and 5). Another group of virulence determinants previously implicated in bacterial shedding are fimbrial adhesins of serotype Typhimurium. A serotype Typhimurium *agf pef fim lpf* mutant is recovered in significantly lower numbers from fecal pellets at 5 days postinfection than the isogenic wild type during a competitive infection experiment (58). Thus, inactivation of genes required for attachment to or invasion of the intestinal mucosa may result in reduced bacterial shedding. While attachment of serotype Typhimurium to the murine small intestine can be detected using ligated ileal loops (7, 9), mutational inactivation of *shdA* did not reduce bacterial numbers recovered from this model (Fig. 6A). In contrast, a serotype Typhimurium *invA* mutant colonized Peyer's patches at reduced levels in the organ culture model, suggesting that bacterial invasion can be detected using this assay (Fig. 6B). These data suggest that unlike mutations in fimbrial biosynthesis or invasion genes, the shedding defect of a *shdA* mutant was not caused by decreased bacterial attachment to or invasion of the mucosa of the murine small intestine but may be due to a different mechanism.

A mutation in *invA* or the simultaneous inactivation of the *agf*, *pef*, *fim*, and *lpf* operons results in a 50- and 26-fold attenuation of serotype Typhimurium for mouse virulence, respectively (25, 58). The attenuating effect of these mutations suggests that the corresponding attachment or invasion genes increase the disease-induced mortality rate, α, which is expected to result in a reduction of the basic case reproductive.
number, \( R_0 \), of serotype Typhimurium (equation 3). At the same time, however, \( invA \), \( agf \), \( pef \), \( pfm \), and \( lpf \) may reduce the clearance rate, \( v \), which would be predicted to increase the basic case reproductive number. It is therefore difficult to predict whether the net result of expressing fimbriae or invasion genes is an increase or a decrease in the basic case reproductive number of serotype Typhimurium. In contrast, mutational inactivation of \( shdA \) did not reduce the disease-induced mortality rate, \( \alpha \), but decreased the duration of shedding (Fig. 4). This phenotype is consistent with a role of \( shdA \) in decreasing the clearance rate, \( v \), thereby resulting in an increase in the basic case reproductive number, \( R_0 \), of serotype Typhimurium (equation 3). The phylogenetic distribution of \( shdA \) and its predicted effect on the basic case reproductive number are consistent with the idea that acquisition of this gene may have contributed to the expansion in host range of \( S. enterica \) subspecies I to include warm-blooded animals.

ACKNOWLEDGMENTS

We are grateful to Renée Tsolis for helpful suggestions on the manuscript and Kenneth Sanderson for providing strains from the SARB and SARC collections.

Work in A.B.’s laboratory is supported by Public Health Service grants AI40124 and AI44170 and grant 9802610 from the U.S. Department of Agriculture.

REFERENCES


FIG. 6. Recovery of bacteria from the intestinal organ culture model. Intestinal loops were infected with an equal mixture of RAK1 (shdA) and IR715 (wild type) (A) or AJB751 (invA) and AJB82 (wild type) (B). The output ratios were determined for Peyer’s patches (PP) and villous intestine (VI). Data were converted logarithmically and are given as means ± standard errors (error bars).