Molecular and Phenotypic Analysis of the CS54 Island of *Salmonella enterica* Serotype Typhimurium: Identification of Intestinal Colonization and Persistence Determinants

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The *shdA* gene is carried on a 25-kb genetic island at centisome 54 (CS54 island) of the *Salmonella enterica* serotype Typhimurium chromosome. In addition to *shdA*, the CS54 island of *Salmonella* serotype Typhimurium strain LT2 contains four open reading frames designated *ratA*, *ratB*, *sivI*, and *sivH*. DNA hybridization analysis revealed that the CS54 island is comprised of two regions with distinct phylogenetic distribution within the genus *Salmonella*. Homologues of *shdA* and *ratB* were detected only in serotypes of *Salmonella enterica* subsp. I. In contrast, sequences hybridizing with *ratA*, *sivI*, and *sivH* were present in *S. enterica* subsp. II and *S. bongori* in addition to *S. enterica* subsp. I. Deletion of the *ratA* and *sivI* genes did not alter the ability of *Salmonella* serotype Typhimurium to colonize the organs of mice. Insertional inactivation of the *sivH* gene resulted in defective colonization of the Peyer’s patches of the terminal ileum but normal colonization of the cecum, mesenteric lymph nodes, and spleen. Deletion of the *shdA* gene resulted in decreased colonization of the cecum and Peyer’s patches of the terminal ileum and colonization to a lesser degree in the mesenteric lymph nodes and spleen 5 days post-oral inoculation of mice. A strain containing a deletion in the *ratB* gene exhibited a defect for the colonization of the cecum but not of the Peyer’s patches, mesenteric lymph nodes, and spleen. The *shdA* and *ratB* deletion strains exhibited a shedding defect in mice, whereas the *sivH* deletion strain was shed at numbers similar to the wild type. These data suggest that colonization of the murine cecum is required for efficient fecal shedding in mice.

*Salmonella*-induced enterocolitis is currently the leading food-borne illness with a lethal outcome in the United States (33). The causative agents, nontyphoidal serotypes of *Salmonella enterica* subsp. I, are introduced into the human food supply primarily because these pathogens persist within populations of livestock and domestic fowl (11, 13, 15, 17–19, 44). Fecal contamination of the environment is the factor most important for the transmission of *S. enterica* subsp. I. Serotypes among animals on the farm and during transport (14, 16, 32, 45, 46). Slaughter of infected animals and the subsequent contamination of food products which may occur during processing explains the high prevalence of *S. enterica* subsp. I. Serotypes in meat and meat products in the United States (41).

*Salmonella bongori* and *Salmonella enterica* subsp. II, IIIa, IIIb, IV, VI, and VII can cause intestinal and extraintestinal infections in humans with symptoms that are indistinguishable from those resulting from infection with nontyphoidal serotypes of *S. enterica* subsp. I (1). However, human cases of infection with *S. bongori* or *S. enterica* subsp. II, IIIa, IIIb, IV, VI, and VII are rare (1) because these pathogens are mainly associated with cold-blooded vertebrates and are infrequently isolated from livestock, domestic fowl, or derived food products (38). These data suggest that serotypes of *S. enterica* subsp. I possess genetic determinants that enable them to persist in the intestines of livestock and domestic fowl but which are absent from serotypes of *S. bongori* and *S. enterica* subsp. II, IIIa, IIIb, IV, VI, and VII. Candidates for such genes have been identified by genomic comparison of *Salmonella* serotypes. Hybridization of genomic DNA from *S. bongori*, *S. enterica* subsp. IIIa, and *S. enterica* subsp. I serotypes Paratyphi A and B with a *Salmonella enterica* serotype Typhimurium LT2 microarray and comparison of the complete genome sequences identified a subset of 216 LT2 genes which have close homologues in one or several *S. enterica* subsp. I. Serotypes but are absent from *Escherichia coli* K-12, *Klebsiella pneumoniae*, *S. enterica* subsp. IIIa, and *S. bongori* (31, 39). Porwollik et al. point out that only 88 of these genes, including *bigA, envF, sifAB, srfI, srgAB, saf, stb, stc, std, sif, stl, and shdA*, are named, which indicates that this group of genes has remained largely unstudied (39).

It was recently shown that one of these genes, *shdA*, is required for persistent shedding of *Salmonella* serotype Typhimurium with the feces from orally inoculated mice (29). The ShdA protein of *Salmonella* serotype Typhimurium, a member of the autotransporter family, is an outer membrane protein that binds fibronectin (28). The *shdA* gene is located on an approximately 25-kb island in the *xseA-yfgK* intergenic region of *Salmonella* serotype Typhimurium (31) at centisome 54 (CS54 island). The phylogenetic distribution of *shdA* and its role in the ability of *Salmonella* serotype Typhimurium to be shed with feces raises the question as to whether other genes

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carried on the CS54 island are required for intestinal persistence.

Here we describe the molecular characterization of the CS54 island of *Salmonella* serotype Typhimurium strain ATCC 14028. We investigate the extent of the subsp. I-specific DNA region and the contribution of the carried genes to the colonization of the mouse and shedding with feces.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The *Salmonella* reference collections B and C have been described previously (9, 10). *Salmonella* serotype Typhimurium strain IR715 is a virulent, nalidixic acid-resistant derivative of strain ATCC 14028 and has been described previously (43). *E. coli* strains S17-1 *pir* and DH5α have been described previously (22, 42). Strains were routinely cultured aerobically at 37°C in Luria-Bertani (LB) broth supplemented with antibiotics as appropriate at the following concentrations: carbenicillin, 100 mg/liter; tetracycline, 30 mg/liter; kanamycin, 100 mg/liter; and nalidixic acid, 50 mg/liter. For the detection of tetracycline, 20 mg/liter; chloramphenicol, 30 mg/liter; kanamycin, 100 mg/liter; and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal) 0.1% sodium dodecyl sulfate (31). Labeling of DNA was achieved with the hsp60 expression, 20 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal) per liter was added to LB agar plates. For counter selection of the sacRB marker, bacteria were grown in sucrose broth as described previously (27). The bacteriophage P22 HT105/1 int mutant was used for generalized transduction of antibiotic resistance markers between *Salmonella* serotype Typhimurium strains as previously described (3).

**Cloning and sequence determination of the S. enterica serotype Typhimurium *sxeA* and *yfgK* intergenic region.** The cloning of the 3′ terminus of the *sxeA* gene and the *shdA* gene on the recombinant cosmid pRK284 has been previously described (29). Recombinant clones pRA59, pRA64, pRA71, and pRA73 were derived from pRK284 by subcloning restriction fragments into vectors pUC18 or pHblue-scriptKS(–) in *E. coli* strain DH5α. The complete nucleotide sequence in the CS54 island was determined by using an AutoRead sequencing kit (Pharmacia) and an ALF automated sequencer. The nucleotide sequences were analyzed with the MacVector, version 6.0.1, software package (Oxford Molecular Group).

**Southern hybridization.** Isolation of genomic DNA, digestion with restriction enzymes, and Southern transfer of DNA onto a nylon membrane were performed as previously described (2). Hybridization was performed at 65°C in solutions without formamide. Two 15-min washes were performed under non-stringent conditions at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate. Labeling of DNA probes with fluorescein-11-dUTP and detection with anti-fluorescein antiserum were performed with the Gene Images labeling and detection kit (Amerham-Pharmacia).

**Construction of Salmonella serotype Typhimurium strains with deletions of shdA, ratB, ratA, *sivL*, and *sivH*.** To construct a strain carrying a deletion of the *shdA* gene (bp 5 to +6090) (Fig. 1) DNA regions flanking the *shdA* open reading frame (ORF) were amplified with the primers 5′-GGCGGCGTAGATGGAAGATACC-3′, 5′-GAAGATCTCAGGCCGAGCAGCC-3′, 5′-GGAGGTCTCAATGCCTG-3′, and 5′-GGCGGCGTAGATGGAAGATACC-3′. Reaction products of the predicted size were digested with BglII and ligated by using the rapid DNA ligation kit (Roche) and cloned into the pCR2.1 vector (TA-cloning kit; Invitrogen) in *E. coli* strain DH5α. The resulting plasmid was designated pAH20. The *XbaI* HindIII insert of pAH20 was subcloned into vector pUC18, yielding pAH24, and a BamHI-restricted kanamycin resistance cassette (KSAC, Pharmacia) was cloned onto the BglII site to give rise to plasmid pAH32. The entire insert of pAH32, consisting of the flanking region of *shdA* with a kanamycin resistance cassette, was excised by using NorI and SphI and subcloned into the XbaI/NorI-digested pPE185.2 vector (26) to give rise to plasmid pAH34. Plasmid pAH34 maintained in *E. coli* strain S17-1 *pir* was introduced into *Salmonella* serotype Typhimurium strain IR715 by conjugal transfer, and exconjugants were selected on LB plates supplemented with nalidixic acid and kanamycin. An exconjugant that was resistant to kanamycin but sensitive to chloramphenicol was identified and designated strain AH9 (Fig. 1). Deletion of the *shdA* gene in AH9 was confirmed by Southern hybridization by using a *shdA*-specific probe derived from the pRA38 insert (29) (data not shown).

The construction of a *Salmonella* serotype Typhimurium strain in which the wild-type *shdA* gene was reintroduced into strain AH9 for complementation (strain RAK60) was as follows. A DNA fragment at the 3′ end of the *shdA* ORF was amplified by PCR with primers 5′-GCTCTAGAAGCCGGCTAGACATACACC-3′ and 5′-CCGATATCACATATTTCGACGGCGCTC-3′. The PCR product was digested with XbaI and Smal, ligated into pFUSE (4) previously restricted with XbaI and Smal, and transformed into *E. coli* strain S17-1 *pir* to yield plasmid pRA133. Plasmid pRA133 was introduced into *Salmonella* serotype Typhimurium strain IR715 by conjugal transfer. Exconjugants that had pRA133 combined into the chromosome were selected on LB plus nalidixic acid and chloramphenicol, and one nalidixic acid-resistant and chloramphenicol-resistant exconjugant was designated strain RAK23. The chromosomal construct was confirmed by Southern hybridization of EcoRV-digested RAK23 genomic DNA by using the pRA133 insert as a probe (data not shown). A bacteriophage P22 HT105/1 int mutant lysate propagated on strain RAK23 was used to transduce the chloramphenicol resistance marker of pRA133 present in the chromosome adjacent to the *shdA* stop codon into strain AH9. Transductants were selected by plating on LB-plus-chloramphenicol plates containing 5 mM EGTA. Cotransduction of the wild-type *shdA* gene into strain AH9 for complementation was tested for by replica plating transductants on LB-plus-chloramphenicol and LB-plus-kanamycin plates. A plasmid P22-sensitive, chloramphenicol-resistant, kanamycin-sensitive transductant was designated RAK60 and purified from contaminating P22 phase by streaking to single colonies twice on Evans blue uracil (EBU) plates (7).

To construct a strain carrying a deletion of the *ratA* gene (−30 to +5996) (Fig. 1), DNA regions flanking the *ratA* ORF were amplified with the primers 5′-GTTGAGGGTGGTAAAAATCACGTC-3′ and 5′-GGGCGCTGATGGCGGATCG-3′. Reaction products of the predicted size were digested with BglII, ligated by using the rapid DNA ligation kit (Roche), and cloned into the pCR2.1 TOPO vector (TA-cloning kit; Invitrogen) in *E. coli* TOP10 cells. The resulting plasmid was designated pAH10. The *XbaI* HindIII insert from pAH10 was subcloned into vector pUC18, yielding pAH18. A BamHI-restricted kanamycin resistance cassette (KSAC, Pharmacia) was cloned into the BglII site to give rise to plasmid pAH31. The SpeI/NorI insert from pAH31 was subcloned into the suicide vector pEP185.2, yielding plasmid pAH33. Plasmid pAH33 maintained in *E. coli* strain S17-1 *pir* was introduced into

**FIG. 1.** ORFs of the CS54 island of *Salmonella* serotype Typhimurium strain ATCC 14028 and deletion or insertion mutations constructed for phenotypic analysis. ORFs are indicated by arrows. The position of a dispersed repeat (cross-hatched bar) and remnant of an IS element (open bar) are indicated. The lengths (in base pairs) of the ORFs are indicated from the ATG codon (+1) to the stop codon. The position of the deletion or insertion in *shdA*, *ratB*, *ratA*, *sivL*, or *sivH* in strains AH9, AH12, AH8, AH10, and RAK19 are indicated.
Salmonella serotype Typhimurium strain IR715 by conjugal transfer, and exconjugants were selected on LB plus nalidixic acid and chloramphenicol. Plasmid pRDH10 carries the sacRB locus, which may be used as a counter selectable marker during growth in the presence of 5 mM EGTA and in the absence of NaCl at 30°C. In order to select for the loss of pRDH10 from the chromosome due to a recombination between homologous DNA derived from the chromosome and pAH40, exconjugants were grown in sucrose broth for 18 h at 30°C with shaking. Serial 10-fold dilutions were plated on sucrose plates and incubated at 30°C for 18 h. Colonies resulting from these plates were replica plated on LB plus nalidixic acid and LB chloramphenicol, and several chloramphenicol-resistant-selectable variants were selected. To distinguish between variants that had lost the pRDH10 plasmid from the chromosome by homologous recombination resulting in regeneration of the wild-type ralB locus from those resulting in the introduction of a deletion in the ralB locus, primers 5′ TTAAGCATGATGCGCTGCTGCC 3′ and 5′ GAA GATCTTCAATACCGCTATCCGTTTGGG 3′ were used to amplify the sequence across the deletion. One resolved exconjugant was designated AH12. The deletion of ralB in strain AH12 was confirmed by Southern hybridization by using a probe constructed from plasmid pAH17 (data not shown).

The construction of a Salmonella serotype Typhimurium strain in which the wild-type ralB gene was reintroduced into strain AH12 for complementation (strain RAK8) was as follows. A DNA fragment at the 3′ end of the ralB ORF was amplified by PCR with primers 5′ GAGAAGATCCCGACGCGCTCTCA GAC 3′ and 5′ GGGCGGTAGTAGGAGATAC 3′. The PCR product was digested with XbaI and SacI and ligated into pPG704 (26) previously restricted with XbaI and SacI and transformed into E. coli S17-1pir to yield plasmid pMAR4. Plasmid pMAR4 was introduced into Salmonella serotype Typhimurium strain IR715 by conjugal transfer. Exconjugants that had pMAR4 recombined into the chromosome were selected on LB plus nalidixic acid and carbenicillin, and one nalidixic acid-resistant and carbenicillin-resistant exconjugant was designated strain MRZ4. The chromosomal construct was confirmed by Southern hybridization (data not shown). A bacteriophage P22 HT105/1 int mutant lysate propagated on strain MRZ4 was used to transduce the chloramphenicol resistance marker of pMAR4 present in the chromosome adjacent to the ralB::Kmr allele from strain ST59 into strain IR715. A phage P22-sensitive, carbenicillin-resistant, kanamycin-sensitive transductant was designated RAK16 and purified by sequential plating of P22 phage by streaking to single colonies twice on EBU plates (7). For competitive infection experiments, groups of 5 mice were infected by oral gavage with an approximately 1:1 mixture of mutant and isogenic parents at a dose of approximately 10^9 CFU/mouse. Fecal pellets were homogenized in 1 ml of phosphate-buffered saline. The cecum, 3 Peyer's patches of the terminal ileum adjacent to the cecum, the mesenteric lymph nodes, and the spleen were harvested serial 10-fold dilutions of fecal pellets and homogenized organs were plated on LB plates containing the appropriate antibiotics for competitive infection experiments with
strains AJB715 and AH12, LB-plus-nalidixic-acid agar plates were supplemented with XP to distinguished between colonies expressing PhoN (AH12) and colonies that were PhoN negative (AJB715). Data were normalized by dividing the output ratio (CFU of the mutant/CFU of the wild type) by the input ratio (CFU of the mutant/CFU of the wild type). In case only one bacterial strain was recovered from fecal pellets, the limit of detection was determined for the missing strain that were PhoN negative (AJB715). Data were normalized by dividing the output with XP to distinguished between colonies expressing PhoN (AH12) and colonies strains AJB715 and AH12, LB-plus-nalidixic-acid agar plates were supplemented with XP to distinguished between colonies expressing PhoN (AH12) and colonies that were PhoN negative (AJB715). Data were normalized by dividing the output ratio (CFU of the mutant/CFU of the wild type) by the input ratio (CFU of the mutant/CFU of the wild type). In case only one bacterial strain was recovered from fecal pellets, the limit of detection was determined for the missing strain that were PhoN negative (AJB715). Data were normalized by dividing the output with XP to distinguished between colonies expressing PhoN (AH12) and colonies strains AJB715 and AH12, LB-plus-nalidixic-acid agar plates were supplemented with XP to distinguished between colonies expressing PhoN (AH12) and colonies that were PhoN negative (AJB715). Data were normalized by dividing the output ratio (CFU of the mutant/CFU of the wild type) by the input ratio (CFU of the mutant/CFU of the wild type). In case only one bacterial strain was recovered from fecal pellets, the limit of detection was determined for the missing strain that were PhoN negative (AJB715). Data were normalized by dividing the output with XP to distinguished between colonies expressing PhoN (AH12) and colonies strains AJB715 and AH12, LB-plus-nalidixic-acid agar plates were supplemented with XP to distinguished between colonies expressing PhoN (AH12) and colonies that were PhoN negative (AJB715).

## RESULTS

### Nucleotide sequence analysis of the CS54 island

The \textit{shdA} gene is carried at one end of a genetic island present in serotypes of \textit{S. enterica} subsp. I. But absent from serotypes of \textit{S. enterica} subsp. II to VII and the closely related species \textit{S. bongori} and \textit{E. coli} (29). In order to investigate the extent of the subsp. I-specific genetic island, the complete nucleotide sequence of the insert of pRK824, a cosmid from a gene bank of \textit{Salmonella} serotype Typhimurium strain ATCC 14028, was determined and deposited in the GenBank database (accession no. AF140550). From the stop codon of \textit{xseA}, which is down-stream of \textit{shdA} and defines one border of the genetic island, a DNA sequence with no significant homology to the \textit{E. coli} K-12 MG1655 genome nucleotide sequence extended for 24,030 bp. Following this was an ORF, carried on the reverse strand, whose deduced amino acid sequence exhibited 97.5% identity over the terminal 40 amino acids of YlgK of \textit{E. coli} K-12 (Fig. 1). The ORFs of the CS54 island of \textit{Salmonella} serotype Typhimurium strain ATCC 14028 were identical to those of \textit{Salmonella} serotype Typhimurium strain LT2 (31), except that \textit{ratB} in ATCC 14028 was truncated by a point mutation. Four ORFs were carried on the CS54 island in addition to \textit{shdA} (Fig. 1A). The properties of these potential coding sequences are summarized in Table 1. The GC content of the island varied between 28%, in the \textit{shdA-ratB} intergenic region, and 59.9%, in the \textit{shdA}, \textit{ratA}, and \textit{ratB} ORFs. The \textit{shdA-ratB} intergenic region contained a 134-bp sequence with homology to dispersed repeat sequences (93 to 69% identity) that are repeated 15 times in the \textit{E. coli} K-12 MG1655 genome (6). No function has been assigned to these \textit{E. coli} repeats. The \textit{shdA-ratB} intergenic region also contained a partial ORF with homology (84% over 26 amino acids) to the terminal 26 residues of the IS1 element from \textit{Shigella dysenteriae} (accession no. P03832) (35).

### Distribution of the CS54 island within the genus Salmonella

Hybridization analysis with Southern blots was employed to determine the distribution of sequences in the CS54 island of \textit{Salmonella} serotype Typhimurium by using a collection of serotypes representing the full range of genetic variation within the genus \textit{Salmonella}. This collection comprised 21 isolates from \textit{S. enterica} subsp. I (from the \textit{Salmonella} reference collection B), representing 17 different serotypes, and 14 isolates from \textit{S. enterica} subsp. II, IIIa, IIIb, IV, VI, and VII and \textit{S. bongori} (from the \textit{Salmonella} reference collection C). The distributions of the \textit{xseA} and \textit{shdA} genes within this collection have been previously described (29). We extended the hybridization analysis upstream from the \textit{shdA} gene by using nucleic Acid sequence accession number.

The complete nucleotide sequence of the insert of pRK824 was determined and deposited in the GenBank database under accession no. AF140550.

### TABLE 1. ORFs of the \textit{xseA}-\textit{yfgK} intergenic region of \textit{Salmonella} serotype Typhimurium strain ATCC 14028

| Gene designation | % GC content | Peptide length<sup>a</sup> | Peptide mass (kDa) | Signal peptide<sup>b</sup> | Protein description |
|------------------|--------------|---------------------------|-------------------|---------------------------|---------------------|
| \textit{shdA}    | 58.0         | 2,035                     | 207               | 1–60                      | Similar to MisL (35%), AIDA-I (34%), IcsA (30%) (carboxy terminus) |
| \textit{ratB}    | 59.9         | 1,947                     | 204               | 1–27                      | Similar to RatA     |
| \textit{ratA}    | 59.0         | 1,865                     | 200               | 1–30                      | Similar to RatB     |
| \textit{sivI}    | 53.3         | 319                       | 33.9              | 1–28                      | Similar to SivH (34.5%) (over terminal 110 residues) |
| \textit{sivH}    | 52.3         | 730                       | 81.2              | 1–19                      | Similar to invasin (49.5%), intimin (48%) (amino terminus) |

<sup>a</sup> Given as the number of amino acid residues.

<sup>b</sup> Amino acid residue positions are given.
FIG. 2. Position and alignment of the imperfect repeats of RatA and RatB. In the Clustal alignment of the imperfect repeats (top), dark shaded boxes indicate identical residues, light shaded boxes indicate residues with similar biochemical properties, and dashes indicate gaps in alignment. The positions of the repeats in the RatA and RatB proteins (arrows) are indicated (filled bars) (bottom).
Typhimurium (Tm1)
Paratyphi B (Pb5)
Muenchen (Mu1)
Paratyphi B (Pb1)
Paratyphi B (Pb3)
Paratyphi A (Pa1)
Sendai (Se1)
Gallinarum (Ga1)
Pullorum (Pu3)
Pullorum (Pu4)
Heidelberg (He1)
Dublin (Du1)
Enteritidis (En1)
Choleraesuis (Cs1)
Choleraesuis (Cs11)
Paratyphi C (Pc2)
Typhisuis (Ts1)
Agona (Ag1)
Derby (De1)
Typhi (Tp1)
Montevideo (Mo1)

FIG. 3. Phylogenetic distribution of the ratB, ratA, sivI, and sivH genes within the genus Salmonella. Southern blot analysis with representative serotypes of S. enterica (subspecies are indicated in roman numerals) and S. bongori (S.b.) is shown. Genomic DNA prepared from serotypes indicated on the left (strain designations are indicated in parentheses) was hybridized with DNA probes pRA59, pRA64, pRA71, and pRA73. The locations of these DNA probes (closed bars) relative to the ORFs of the CS54 island (arrows) are indicated on the map shown at the top.

acid probes generated from the pRK824 restriction fragments shown in Fig. 3. A probe overlapping the ratB gene (pRA59) hybridized with genomic DNA of all serotypes of S. enterica subsp. I (except one isolate [Cs11] of S. enterica subsp. I serotype Choleraesuis) but not with serotypes from S. enterica subsp. II to VII or S. bongori. In contrast, probes specific for ratA (pRA64) and sivH (pRA71) hybridized with genomic DNA from serotypes of S. enterica subsp. II, S. bongori, and all
serotypes of *S. enterica* subsp. I. No signal was detected with genomic DNA prepared from serotypes of *S. enterica* subsp. IIIa, IIIb, IV, VI, or VII. The DNA probe specific for *yfgK* (pRA73) hybridized with serotypes from all lineages of the genus *Salmonella*, indicating the position of the right border of the CS54 island.

In summary, these data indicated that *shdA*, *ratA*, *ratB*, *sivI*, and *sivH* are carried by all serotypes of *S. enterica* subsp. I tested, with the notable exception of the absence of *ratB* from the serotype Choleraesuis isolate Cs11. While *shdA* and *ratB* were absent from non-*S. enterica* subsp. I serotypes, *sivH*, *sivI*, and *ratA* hybridizing sequences were detected in serotypes of *S. enterica* subsp. II and *S. bongori*. The presence of *sivH*, *sivI*, and *ratA* in *S. enterica* subsp. II and *S. bongori* serotypes in addition to *S. enterica* subsp. I suggested a complex evolutionary history of the CS54 island, involving multiple horizontal transfer or deletion events.

**Contribution of the genes carried on the CS54 island to organ colonization of the BALB/c mouse.** Inbred mouse strains that are genetically susceptible to *Salmonella* serotype Typhimurium infection are frequently used to model typhoid fever caused by *S. enterica* serotype Typhi. Strains of *Salmonella* serotype Typhimurium were constructed in which *shdA* (strain AH9), *ratA* (strain AH8), *ratB* (AH12), *sivI* (strain AH10), or *sivH* (strain RAK19) were deleted or interrupted by insertion of a kanamycin resistance cassette (Fig. 1). The phenotypes of these strains were characterized by using competitive infection experiments with the inbred mouse strain BALB/c.

We previously reported the phenotype of a *Salmonella* serotype Typhimurium strain RAK1, in which the *shdA* gene was interrupted by the chloramphenicol acetyltransferase (*cat*) gene following oral inoculation of BALB/c mice. To further characterize the *shdA* colonization defect, we constructed a strain with a precise deletion of the *shdA* ORF (AH9) by allelic exchange (Fig. 1). I suggested a complex evolutionary history of the CS54 island, involving multiple horizontal transfer or deletion events.

**FIG. 4.** Recovery of bacteria from the cecum, Peyer’s patch, mesenteric lymph node (MLN), and spleen of BALB/c mice 5 days post-oral inoculation with an equal mixture of AH9 (Δ*shdA::*Kmr, open bars) or RAK60 (AH9 complemented, filled bars) and IR715 (wild type [wt]) (A), AH12 (Δ*ratB*, open bars) or RAK38 (AH12 complemented, filled bars) and AJB715 (wt) (B), AH8 (Δ*ratA::*Kmr) and IR715 (wt) (C), AH10 (Δ*sivI::*Kmr) and IR715 (wt) (D), or RAK19 (Δ*sivH::*Kmr open bars) or RAK59 (RAK19 complemented, filled bars) and IR715 (wt) (E). The ratio of the two strains present is given as the mean ± standard error. An asterisk indicates that the output ratio was significantly different (*P* < 0.05) from that present in the inoculum.
The colonization defect was greater in the cecum and Peyer’s patches of the ileum (50- to 100-fold more CFU of IR715 than of AH9) than in the mesenteric lymph nodes and spleen (5- to 10-fold more CFU of IR715 than of AH9).

In order to determine whether other genes carried on the CS54 island have functions related to that of shdA, we characterized strains AH12 (ΔratB), AH8 (ΔratA::Km'), AH10 (ΔsivI::Km'), and RAK19 (sivH::Km') in competitive infection experiments with the wild type (IR715). Four mice were each inoculated orally with an equal mixture of IR715 and either AH10 (ΔsivI::Km'), AH8 (ΔratA::Km'), AH12 (ΔratB), or RAK19 (sivH::Km'). Five days postinoculation, the number of CFU of the wild type and mutant in homogenates of the cecum, Peyer’s patches, mesenteric lymph nodes, and spleen were determined (Fig. 4B to E). Strains AH8 (ΔratA::Km') and AH10 (ΔsivI::Km') did not exhibit a colonization defect in the cecum, Peyer’s patches, mesenteric lymph nodes, or spleen (Fig. 4C and D). RAK19 (ΔsivH::Km') was not recovered from the Peyer’s patches of the terminal ileum (the limit of detection was 5 CFU), indicating that IR715 was present in this tissue at >10-fold-greater numbers than it was in RAK19 (Fig. 4E). IR715 (wild type) was recovered in significantly (P < 0.005) greater numbers (40-fold) from the cecum than was strain AH12 (ΔratB) (Fig. 4B). These data suggested that RatB is required for the colonization of the cecum while SivH contributes to the colonization of the Peyer’s patches.

The role of shdA, sivH, and ratB in long-term shedding from CBA/J mice. A Salmonella serotype Typhimurium shdA aroA mutant was used previously to investigate the role of the shdA gene in fecal shedding of bacteria from BALB/c mice at time points beyond day 5 postinoculation (29). The introduction of an aroA mutation was necessary in these experiments, since BALB/c mice show signs of lethal morbidity when infected with virulent Salmonella serotype Typhimurium within 4 to 6 days while the shdA phenotype becomes most pronounced at later times postinoculation. To study the role of shdA under more natural conditions (i.e., in a fully virulent Salmonella serotype Typhimurium strain background) we used a resistant mouse lineage, CBA/J. This strain does not succumb to infection by Salmonella serotype Typhimurium, but the intestine becomes colonized at a high level (10⁴ to 10⁵ CFU/mg of feces) for several weeks (34). To assess the effect of a mutation in shdA on bacterial shedding, 8 mice were inoculated orally with an equal mixture of AH9 (ΔshdA::Km') and IR715 (wild type). The presence of each strain in fecal pellets was enumerated over a 42-day period (Fig. 5A). The mean ratio of AH9 to IR715 was not significantly different from the inoculum ratio (1:1) in the first 19 days postinoculation, with the exception of one occasion (day 5 postinoculation) in which AH9 (ΔshdA::Km') was recovered in significantly (P < 0.05) greater numbers (threefold) than IR715 (wild type). On day 21 and on subsequent days postinoculation, IR715 (wild type) was recovered in significantly (P < 0.05) greater numbers (10- to 500-fold) than AH9 (ΔshdA::Km'). These results were consistent with data reported previously that a Salmonella serotype Typhimurium shdA aroA mutant is recovered at lower numbers from fecal pellets of BALB/c mice at late time points postinoculation than an isogenic Salmonella serotype Typhimurium aroA mutant (29).

We next characterized the shedding phenotype of Salmonella serotype Typhimurium strains containing deletions of the sivH gene or the ratB gene in groups of 6 or 9 CBA/J mice, respectively, during competitive infection experiments with the Salmonella serotype Typhimurium wild type (IR715). The mean ratio of RAK19 to IR715 was not significantly different from the inoculum ratio (1:1) on any day, with the exception of day 1 postinoculation, at which point RAK19 (sivH::Km') was recovered in significantly (P < 0.05) greater numbers (twofold) than IR715 (wild type) (Fig. 5B). In contrast, the ratio of AH12 to IR715 decreased over time. On day 5 postinoculation, and on all subsequent days investigated, significantly (P < 0.05) greater numbers of AJB715 CFU were shed with the feces than CFU of the ratB mutant (AH12) (Fig. 5C). On day 17 postinoculation and on subsequent days, AJB715 was recovered in >1,000-fold-higher numbers than AH12 (ratB). These results
showed that, in contrast to a mutation in sivH, a deletion of the ratB gene reduced the magnitude of fecal shedding of Salmonella serotype Typhimurium from mice.

Correlation of cecal colonization and fecal shedding. The characterization of the role of CS54 island-borne genes in the colonization of BALB/c mice and shedding with the feces of CBA/J mice revealed a correlation between the role of cecal colonization in BALB/c mice and shedding with the feces following oral inoculation of CBA/J mice. However, these observations were made in different inbred mouse strains. To further study this correlation, we investigated the role of the shdA and ratB genes in fecal shedding and organ colonization in groups of 5 CBA/J mice. The colonization defect was observed in the cecum and fecal pellets (Fig. 6A). The greatest colonization defect was observed in the cecum and fecal pellets 21 days postinfection, at which stage the wild-type strain (IR715) was recovered in approximately 100-fold-higher numbers than the wild type (Fig. 7). The shedding defect observed for the ΔshdA mutant (AH9) and the ΔratB mutant (AH12) during competitive infection with the wild type correlated with the colonization defect exhibited in the cecum. Significantly greater numbers of wild-type CFU were recovered in the cecum than were CFU of the shdA and ratB mutants. In contrast, the sivH mutant was recovered in numbers similar to those of the wild type (Fig. 7). The shedding defect observed for the ΔshdA mutant (AH9) and either the ΔsivH mutant (AH9), or the ΔsivH mutant (RAK19). The CFU of Salmonella serotype Typhimurium strains in approximately 100 mg of feces were enumerated on days 1, 3, and 5 postinoculation and in the cecum and Peyer's patches on day 5 postinoculation. On day 5 postinoculation, significantly greater numbers of wild-type CFU were recovered in the feces than were CFU of the shdA and ratB mutants. In contrast, the sivH mutant was recovered in numbers similar to those of the wild type (Fig. 7).

DISCUSSION

A common feature of many pathogenicity islands of enteric pathogens is their insertion at tRNA loci (23). For example, the selC tRNA locus is the site of SPI-3 integration in Salmonella serotype Typhimurium (5) and the site of the LEE pathogenicity island integration in enteropathogenic E. coli strains (37). This was not the case for the CS54 islands of Salmonella serotype Typhimurium. Instead, the island was found to be carried between the xseA gene, which encodes the exonuclease.
VII subunit, and the yfgK gene, whose function is currently unknown. Atypical GC content (often low GC content) is frequently an indication of horizontal acquisition (23). The shdA, ratB, and ratA genes have a G+C content of between 58.0 and 59.9%, which is higher than the average G+C content of 53% determined for the Salmonella serotype Typhimurium genome (31). The sinI and sinH ORFs, on the other hand, have a G+C content which is similar to the genomic average. The presence of remnants of an insertion element in the shdA-ratB intergenic region suggests that mobile genetic elements may be responsible for the current distribution of the CS54 island within the genus Salmonella.

We describe the complete coding sequence of the CS54 island of Salmonella serotype Typhimurium strain ATCC 14028. Including the previously described shdA locus, a total of 5 ORFs and the remnants of an insertion element were identified in the region. The sequence of strain 14028 differed from that determined for Salmonella serotype Typhimurium strain LT2 in that the ratB ORF was truncated by a point mutation. As a result, RatB encoded by strain LT2 is predicted to be composed of eight imperfect direct repeats of approximately 200 amino acids while RatB of strain 14028 contains just the first six of these repeats (Fig. 2).

The absence of the CS54 island from the E. coli K-12 genome suggested that this region was acquired by horizontal transfer or lost from the E. coli lineage by deletion. The shdA gene was previously reported to be present in serotypes of S. enterica subs. I and absent from S. enterica subs. II to VII and S. bongori (29). Hybridization of genomic DNA from one S. enterica subs. IIIa isolate and one S. bongori isolate with an DNA array of Salmonella serotype Typhimurium strain LT2 indicates that the ratB gene is absent from both strains while sinI is present in S. bongori but absent from S. enterica subs. IIIa (31). The distribution of ratA and sinH was not previously investigated since the LT2 DNA array of McClelland and coworkers does not contain PCR products specific for these ORFs (31). Here, we show that the ratB ORF exhibited an identical distribution within the genus to shdA. However, Southern blot analysis suggested a wider distribution of shdA and ratB, as homologous sequences were detected in S. enterica subs. II and S. bongori serotypes in addition to serotypes of S. enterica subs. I (Fig. 3). Together, these data suggest a complex evolutionary history of the CS54 island involving multiple horizontal transfer and/or deletion events.

The phylogenetic distribution of shdA and ratB is of significance because serotypes of S. bongori or S. enterica subs. II, IIIa, IIIb, IV, VI, and VII are mainly reptile associated while serotypes of S. enterica subs. I are frequently isolated from livestock and domestic fowl (38). The analysis of genes present in serotypes of S. enterica subs. I but absent from serotypes of S. bongori and S. enterica subs. II, IIIa, IIIb, IV, VI, and VII may provide an opportunity to gain new insights into mechanisms required for persistence of food-borne pathogens in populations of livestock and domestic fowl. Analysis of fecal samples shows that between 1 and 6% of apparently healthy food animals in the United States shed S. enterica subs. I serotypes with their feces (11, 15, 17–19, 44). Before slaughter, the prevalence of S. enterica subs. I serotypes in fecal samples or the intestine increases further, with 10 to 15% of animals testing positive (13, 44). A body of evidence shows that the main factor responsible for spreading the infection among animals on the farm or among animals during transport to slaughter is fecal contamination of the environment (14, 16, 25, 32, 45, 46). These investigations suggest that fecal shedding of S. enterica subs. I serotypes is of prime importance for persistence of these pathogens within populations of livestock and domestic fowl. It is therefore significant that shdA and ratB, two genes whose presence is restricted to serotypes of S. enterica subs. I, both contributed to the ability of Salmonella serotype Typhimurium to be persistently shed with the feces of mice (Fig. 5). It is interesting in this context that a Salmonella serotype Typhi vaccine strain colonizes and persists in the feces of experimentally infected human subjects at a lower level than a Salmonella serotype Typhimurium vaccine strain, although both vaccine strains were attenuated by the same mutation (24). It remains to be seen whether reduced persistence of the Salmonella serotype Typhi vaccine strain is related to the fact that shdA and ratB are pseudogenes in this serotype (36).

Isolation of a Salmonella serotype from the feces is com-
monly accompanied by bacterial enteritis from the host to ensure transmission by the fecal-oral route is not well defined. Studies with *S. enterica* subspp. I serotype Enteritidis in the mouse show that the bulk of luminal bacteria is localized in the cecum, suggesting that this organ is an important reservoir for fecal shedding in this animal model (12). Mutations in shdA, ratB, and shv resulted in a reduced ability of *Salmonella* serotype Typhimurium to colonize intestinal tissues in BALB/c mice (Fig. 4). Inactivation of shv resulted in a reduced ability to colonize Peyer’s patches (Fig. 4) but did not alter bacterial numbers shed with the feces of CBA/J mice over a period of 44 days (Fig. 5). Similarly, a mutation in invA resulted in a reduced ability of *Salmonella* serotype Typhimurium to colonize Peyer’s patches (3, 21), but this mutation does not alter the ability of the pathogen to be shed with the feces of mice (29). In contrast, mutations in shdA and ratB both reduced the ability of *Salmonella* serotype Typhimurium to colonize the cecum (Fig. 4 and 6), and both mutations resulted in a significant reduction of bacterial numbers shed with the feces of mice over a period of 42 days (Fig. 5). These data further support the notion that the cecum is the main reservoir for fecal shedding of *Salmonella* serotypes from mice.

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