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Citation for published version:

Digital Object Identifier (DOI):
10.1128/IAI.00960-08

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

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Identification of *Salmonella enterica* Serovar Dublin-Specific Sequences by Subtractive Hybridization and Analysis of Their Role in Intestinal Colonization and Systemic Translocation in Cattle

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Received 31 July 2008/Returned for modification 16 August 2008/Accepted 4 September 2008

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† Supplemental material for this article may be found at http://iai.asm.org.

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There are over 2,500 different serovars of *Salmonella enterica*, and some are significant pathogens of animals and humans. All *S. enterica* serovars are closely related, and comparisons of housekeeping genes show 96 to 99.5% sequence identity (reviewed in reference 13). Although *S. enterica* serovars are genetically very similar, they differ significantly in biology, particularly in host range and disease spectrum. *S. enterica* serovars may be broadly classified as ubiquitous, host restricted, and host specific (41). In healthy, adult, outbred hosts, ubiquitous serovars, including *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis, are frequently associated with self-limiting intestinal infections in a wide range of phylogenetically distantly related species (38, 43). Host-specific serovars are almost exclusively associated with typhoidal disease in a single species, for example, *S. enterica* serovar Typhi and *S. enterica* serovar Gallinarum in humans and fowl, respectively (2, 12). Serovars which are predominantly isolated from one particular host species but which occasionally cause disease in other host species are classified as host restricted; for example, *S. enterica* serovar Dublin is associated with cattle (38) but sometimes infects pigs and humans. In general, host-specific and host-restricted serotypes tend to be more virulent, causing systemic disease and causing higher mortality rates than ubiquitous serotypes (reviewed in references 3 and 41).

Survivors of systemic salmonellosis sometimes become chronic carriers, thereby facilitating bacterial circulation in host populations (21).

Analysis of the genetic differences responsible for the phenotypic diversity among serovars is currently a major area of *Salmonella* research. Host restriction has occurred by convergent evolution in some instances, as there are cases in which no close phylogenetic relationship exists between serovars adapted to the same host, for example, *S. enterica* serovar Typhi and other human-restricted serovars (24, 35). Conversely, serotypes that are genetically closely related may be adapted to different hosts, for example, *S. enterica* serovar Choleraesuis and *S. enterica* serovar Paratyphi C (35). Adaptation to a particular host species is a complex process that may involve both the acquisition of serovar-specific sequences by lateral gene transfer and gene decay. A number of serovar-specific insertions, deletions, and frameshift mutations have been described previously (4, 15, 24, 26, 30, 39, 40, 46). For example, sequence analysis of the fimbrial genes in several serovars shows that many of the serovars contain frameshifts in one or several of the operons (reviewed in reference 13). Since fimbrial adhesins are involved in interactions with different receptors, this diversity could influence host specificity.
The best characterized of the serovar-specific islands acquired by horizontal transfer is *Salmonella* pathogenicity island-7 (SPI-7) in *S. enterica* serovar Typhi, which encodes the Vi capsular antigen, which is absent from most other serovars (29).

The aim of this study was to investigate the genetic basis of the differential virulence of *S. enterica* serovar Dublin and *S. enterica* serovar Gallinarum in cattle. Previously, we have reported that *S. enterica* serovar Dublin strain SD3246 elicited severe systemic disease following oral inoculation of calves, whereas *S. enterica* serovar Gallinarum strain SG9 was avirulent by this route (28). Differential virulence was not correlated with intestinal invasion or the induction of enteritis (28) but correlated with increased persistence of *S. enterica* serovar Dublin in intestinal mucosa (28) and the ability of *S. enterica* serovar Dublin to translocate to distal sites via the lymphatic system (33). Though the role of known or putative virulence loci in systemic translocation has been assessed (33), traits associated with the differential virulence of *S. enterica* serovar Dublin SD3246 compared to other serovars remain ill defined. It was recently reported that the virulence plasmid of *S. enterica* serovar Dublin contains a unique 10.8-kb region that is absent from the plasmids of *S. enterica* serovars Choleraesuis, Enteritidis, and Typhimurium and contains 16 potential open reading frames (ORFs) (20). We have previously screened 120 mutants with transposon insertions in this unique region of the *S. enterica* serovar Dublin virulence plasmid (SacI fragments C and F), and only one mutant (G19) exhibited reduced virulence for mice (22). The transposon insertion in G19 was in *vagC* and led to uncontrolled expression of the downstream gene *vagD* (32). Other transposon mutants with insertions in *vagC* were fully virulent (32). Thus, it is unlikely that the other genes on this *S. enterica* serovar Dublin-specific plasmid region are required for virulence. A previous microarray study identified DNA sequences that were present in *S. enterica* serovar Typhimurium, Typhi, Paratyphi A, or Enteritidis but absent from either *S. enterica* serovar Dublin or *S. enterica* serovar Gallinarum (30). The significance of these deleted sequences for *S. enterica* serovar Dublin and *S. enterica* serovar Gallinarum is unknown. *S. enterica* serovar Dublin-specific chromosomal regions have not been previously identified. As the genome sequence of *S. enterica* serovar Dublin SD3246 is unknown, we used suppression subtractive hybridization to identify and analyze *S. enterica* serovar Dublin SD3246 chromosomal genes that are absent from *S. enterica* serovar Gallinarum SG9.

**MATERIALS AND METHODS**

**Bacterial strains.** *S. enterica* serovar Dublin SD3246 (18) and *S. enterica* serovar Gallinarum SG9 (45) were isolated from bovine and fowl typhoid, respectively. *S. enterica* serovar Dublin SD3246 is a Vi antigen-negative isolate, and a nalidixic acid-resistant (Nal') derivative with defined virulence in cattle was used (5, 28, 33). Virulent signature-tagged derivatives of these strains and tagged SD3246 mutants lacking SPI-1 and SPI-2 genes have been described previously (33). Another 71 wild-type *Salmonella* isolates were used in this study: these included strains from the United Kingdom, isolated from animals at the Institute for Animal Health, Compton, isolates obtained from the Veterinary Laboratories Agency, United Kingdom, and isolates from *Salmonella* reference collection B (SARB) (6). These consisted of 31 different serovars and subspecies of *S. enterica*, namely, serovars Dublin (14 isolates), 45: :exx (1), Agama (1), Agona (1), Anatum (1), Brandenburg (2), Choleraesuis (5), Choleraesuis variant Decatur (2), Derby (3), Duisburg (2), Emek (2), Enteritidis (4), Haifa (1), Heidelberg (2), Indiana (1), Infantis (2), Miami (2), Montevideo (2), Muenchen (3), Newport (2), Panama (1), Pullorum (1), Reading (1), Rubislaw (1), Saint- paul (1), Senftenberg (1), Stanley (1), Stanleyville (1), Typhimurium (7), and Wien (2) and *S. enterica* subsp. diarizonae (1). All strains were stored as mid-log-phase cultures in Luria-Bertani (LB) medium containing 15% (vol/vol) glycerol at −70°C. Unless otherwise stated, strains were cultured in LB medium at 37°C with the antibiotics ampicillin (100 μg ml−1), kanamycin (Kan; 50 μg ml−1), and Nal (20 μg ml−1) where appropriate.

**General molecular techniques.** Restriction enzymes, GoTaq DNA polymerase, and T4 DNA ligase were purchased from Promega Corporation (Southampton, United Kingdom) or New England Biolabs (Hertfordshire, United Kingdom) and used according to the manufacturer’s recommendations. Oligonucleotide primers were obtained from Sigma Genosys (Poole, United Kingdom) (see Table S1 in the supplemental material). PCR products for sequencing were purified by using QiaQuick PCR purification kits (Qiagen, Crawley, United Kingdom). Genomic DNA from *Salmonella* was prepared by cetyltrimethyl ammonium bromide extraction as described previously (37). DNA probes for Southern hybridization consisted of digoxigenin-labeled PCR products amplified from strain SD3246 with the digoxigenin DNA labeling and detection kit supplied by Roche Molecular Biochemicals (Mannheim, Germany).

**Subtractive hybridization.** Subtractive hybridization was performed by using *S. enterica* serovar Dublin SD3246 genomic DNA as the tester. Driver DNA contained a mix of *S. enterica* serovar Gallinarum SG9 genomic DNA and *S. enterica* serovar Dublin SD3246 plasmid DNA. Both DNA samples were digested with Rsal. The procedure was carried out by using the Clontech PCR-Select bacterial genome subtraction kit (BD Biosciences, Palo Alto, CA) according to the manufacturer’s instructions. PCR products were cloned into pGEM-T Easy (Promega Corporation, Southampton, United Kingdom) and transformed into chemically competent Escherichia coli JM109 cells (Promega Corporation, Southampton, United Kingdom).

**Construction of signature-tagged *S. enterica* serovar Dublin SD3246 mutants.** Uniquely tagged mini-Tn5Km2 mutants of *S. enterica* serovar Dublin SD3246 Nal' with insertions in the sequences identified by subtractive hybridization were created by targeted lambda red recombinase-mediated integration of linear PCR products (31). Compatible tagged transposons were amplified by PCR using primers which incorporate 40-nucleotide gene-specific homology extensions, designed to replace an internal part of the sequence of interest with the transposon. Products were DpnI digested, purified using QiAQuick spin columns (Qiagen, Crawley, United Kingdom) and electroporated into *S. enterica* serovar Dublin SD3246 Na' harboring the lambda red helper plasmid pKD46 following induction of the recombinase with 0.2% (wt/vol) l-arabinose at 30°C (11). Mutants were selected on LB plates containing Nal and Kan at 37°C and cured of pKD46 by growth at 37°C in the absence of ampicillin. Transposon insertion sites were confirmed by PCR analysis. An *S. enterica* serovar Dublin SD3246 mutant with a deletion spanning phoPQ was also created by this method with the use of primers (see Table S1 in the supplemental material) as a control for intramacrophage survival assays.

**DNA sequencing and analysis.** DNA sequencing reactions were performed using the Quickstart kit (Beckman Coulter, High Wycombe, United Kingdom). For the sequencing of the inserts in the subtractive hybridization library, M13For and M13Rev primers were used (see Table S1 in the supplemental material). Sequencing reactions were run on a Beckman-Coulter CEQ8000 sequencer. The BLASTX and BLASTN programs were used to search the NCBI nonredundant sequence database (http://www.ncbi.nlm.nih.gov) and the COLIBASE database (http://colibase.bham.ac.uk) to identify sequence similarities. GLIMMER version 3.02 (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3_0e) was used to predict coding sequences, and InterProScan (http://www.ebi.ac.uk/InterProScan) was used to predict protein domains.

**Calf experiments.** Animal experiments were conducted according to the requirements of the Animal (Scientific Procedures) Act 1986 (license 30/1998) with the approval of the local ethical review committee. Friesian bull calves were reared, housed, and confirmed to be culture negative for *Salmonella* as described previously (28). Calves 25 to 35 days of age were used for infection experiments. For the screening of a pool of 36 signature-tagged mutants following oral inoculation of calves, mutants were separately inoculated into LB broth supplemented with Kan and Nal and incubated overnight at 37°C. The mutants were pooled, and an aliquot was removed for preparation of “input pool” genomic DNA as described previously (19). Two calves were orally inoculated with the pool (approximately 109 CFU per calf) and 10 109 CFU per calf and 10 109 CFU per calf and 10 109 CFU per calf of *S. enterica* serovar Dublin SD3246 Nal' with insertions in the sequences identified by subtractive hybridization were created by targeted lambda red recombinase-mediated integration of linear PCR products (31). Compatible tagged transposons were amplified by PCR using primers which incorporate 40-nucleotide gene-specific homology extensions, designed to replace an internal part of the sequence of interest with the transposon. Products were DpnI digested, purified using QiAQuick spin columns (Qiagen, Crawley, United Kingdom) and electroporated into *S. enterica* serovar Dublin SD3246 Na' harboring the lambda red helper plasmid pKD46 following induction of the recombinase with 0.2% (wt/vol) l-arabinose at 30°C (11). Mutants were selected on LB plates containing Nal and Kan at 37°C and cured of pKD46 by growth at 37°C in the absence of ampicillin. Transposon insertion sites were confirmed by PCR analysis. An *S. enterica* serovar Dublin SD3246 mutant with a deletion spanning phoPQ was also created by this method with the use of primers (see Table S1 in the supplemental material) as a control for intramacrophage survival assays.

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homogenates of tissue collected at necropsy (distal ileal mucosa, draining mesenteric lymph node [MLN], liver, and spleen) were diluted as required and plated onto MacConkey agar containing Kan and NaI to isolate “output pool” bacteria. A sample of jugular blood collected during the cannulation was similarly plated. For each site, ca. 2,500 to 12,000 colonies were pooled for preparation of output pool genomic DNA. Amplification of radiolabeled tags from input and output pools and dot blot hybridizations were performed as described previously (25).

For the determination of competitive indices (CIs) in vivo, bacterial strains were grown in LB broth supplemented with NaI overnight at 37°C. Wild-type and mutant strains were mixed in equal numbers (ca. 1.5 × 10^9 CFU per CFU) in 20 ml antacid and used to inoculate a total of six calves by the oral route (three independent experiments with two calves per experiment). At 3 days postinoculation, an effluent lymphatic vessel was cannulated and lymph was collected as described above. Wild-type and mutant bacteria were enumerated by plating of serial dilutions of the lymph or homogenized tissue samples collected at necropsy onto MacConkey agar with NaI and with NaI plus Kan. The number of wild-type bacteria was determined by subtraction of the count on NaI and Kan medium (mutant) from that on NaI alone. The CI was calculated as the ratio of mutant to wild type in the output pool divided by the ratio of mutant to wild type in the inoculum. Data are presented as the mean CIs ± standard errors of the means. The Mann-Whitney nonparametric test was used to determine whether the output ratio differed significantly from the input ratio. P values of <0.05 were considered significant.

Mouse experiments. For the determination of CIs in mice, bacteria were grown in LB broth supplemented with NaI overnight at 37°C. Wild-type and mutant strains were mixed in equal numbers, and 16 female C57Bl/6 mice (6 to 8 weeks of age) were infected by the oral route via a gavage needle with approximately 2 × 10^6 CFU diluted in phosphate-buffered saline (PBS). Animals were examined at least twice daily. Mice showed symptoms of systemic salmonellosis after 3 to 6 days, at which time they were humanely killed. Spleens and livers were removed, each organ was homogenized in PBS, and serial dilutions of the homogenates were examined at least twice daily. Mice showed symptoms of systemic salmonellosis. In some experiments, bacterial strains were grown overnight at 25°C in complete EMEM containing 100 

RESULTS

Construction and sequence analysis of a library of S. enterica serovar Dublin SD3246 sequences absent from S. enterica serovar Gallinarum SG9. Preliminary results of experiments using total DNA from these two strains to produce a subtractive hybridization library showed that most products contained S. enterica serovar Dublin SD3246 virulence plasmid genes (data not shown). Since S. enterica serovar Dublin-specific plasmid genes are well studied (20, 22, 33), we prepared another subtractive hybridization library by including SD3246 plasmid DNA in the driver DNA sample to identify chromosomal genes present in S. enterica serovar Dublin SD3246 but not SG9. Sequence analysis and BLASTN searches of this library identified 51 clones containing inserts with no significant sequence similarity to the sequenced S. enterica serovar Gallinarum strain (strain 287/91), of which 41 were unique (Table 1).

The COLIBASE database contains sequence data from a partially sequenced S. enterica serovar Dublin strain, CT0201853. The G+C content of this strain is 52.2%, BLASTN searches using the 41 fragments showed that they all had close homologues in CT0201853 (Table 1). The sizes of the 41 subtractive hybridization products ranged from 248 to 1,023 bp, and their G+C contents ranged from 33.9 to 55.6%.

The putative functions of proteins encoded by genes in the subtractive hybridization library were investigated using BLASTX, and many of them were associated with mobile genetic elements, including phage proteins, a transposase, and recombinase hot spot (RHS) elements (Table 1). The phage-related genes (D21 to D41) had G+C contents ranging from

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>G+C Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>D21</td>
<td>Phage protein</td>
<td>33.9%</td>
</tr>
<tr>
<td>D22</td>
<td>Transposase</td>
<td>55.6%</td>
</tr>
<tr>
<td>D23</td>
<td>RHS element</td>
<td>45.2%</td>
</tr>
</tbody>
</table>
41.2 to 54.2% (Table 1). Most of these genes were highly similar to prophage genes from other _S. enterica_ prophages, including the _S. enterica_ serovar Typhimurium prophages Fels-2, Gifsy-2, ST64B, and ST64T. Seventeen of the subtractive hybridization products were not homologous to known phages or other mobile elements (clones D1 to D17) (Table 1). Six were similar to hypothetical proteins, two showed no significant similarities (the D14 and D19) and six were similar to hypothetical homologues to known phages or other mobile elements. Six were identified as _S. enterica_ serovar Paratyphi A ATCC9150.
D16 products), and nine were similar to proteins with known or predicted functions. Most of the last group were present in a number of Salmonella serovars. However, clones D4 and D5 contained inserts that were similar to those of S. enterica serovar Paratyphi A strain ATCC 9150 but were absent from all other sequenced salmonellae except serovar Dublin. The proteins were similar to different regions of the same protein, a putative flagellin structural protein.

Analysis of the distribution of the 41 sequences among sequenced salmonellae using BLASTN showed that seven subtractive hybridization clones (D11, D12, D14, D15, D16, D18, and D30) were present in S. enterica serovar Dublin only, suggesting they might contain sequences unique to S. enterica serovar Dublin. One of these, clone D30, potentially encodes a methyltransferase present on a phage. D18 potentially encodes an RHS element protein and had a relatively low G+C content (39.7%). The two clones whose translated products showed no protein similarities by BLASTX were also potentially specific (39.7%). The two clones whose translated products showed no protein similarities by BLASTX were also potentially specific (39.7%).

The other potentially S. enterica serovar Dublin-specific clone (D15) encoded a product similar to a hypothetical protein from Methanosarcina mazei. Clone D15, which had a low G+C content (35.4%), was also present on contig ABAP01000018, adjacent to a homologue of the S. enterica serovar Typhimurium LT2 Fels-2 phage gene STM2723. Therefore, the S. enterica serovar Dublin-specific sequences in clones D14 and D15 are both inserted into a Fels-2-like prophage.

The other potentially S. enterica serovar Dublin-specific clones (D11 and D12) encoded products with similarity to hypothetical proteins from Enterobacter sp. strain 638. Clone D10 also encoded a protein with similarity to a hypothetical protein from Enterobacter sp. strain 638, though this was also present in S. enterica serovar Enteritidis strains PT4 and LK5. The Enterobacter homologues were analyzed further (see below).

Identification and analysis of SDI-1. As described above, clones D10 to D12 potentially encoded proteins that were similar to Enterobacter sp. strain 638 proteins. The level of amino acid identity ranged from 39 to 75%. Interestingly, three Enterobacter proteins were encoded by genes located close to each other on a region of about 4 kb. In the unfinished S. enterica serovar Dublin genome sequence available at this time, the D10 to D12 sequences were on short, contiguous sequences (contigs 2134 and 2241). Contig 2241 was 2,699 bp, and one end of it was highly homologous to phage tail fiber genes, including S. enterica serovar Typhimurium LT2 STM1049, which encodes the Gifsy-2 phage tail protein (99% nucleotide identity over 928 bp). To determine whether the similarity between S. enterica serovar Dublin and Enterobacter sp. strain 638 extended further than this 4 kb, the sequences of adjacent Enterobacter ORFs were compared with translated sequences in COLIBASE. The Enterobacter proteins Ent638_1030 to Ent638_1051 were similar to the products of 10 translated S. enterica serovar Dublin contigs, suggesting that there was a sequence of about 20 kb of similar organization to an Enterobacter region located adjacent to a phage tail fiber gene. This region showed no nucleotide or amino acid sequence similarity to any other sequenced Salmonella strain, suggesting that it could be an S. enterica serovar Dublin-specific genomic island. Ent638_1052 is a phage tail assembly chaperone and was similar to a number of Salmonella phage tail-associated proteins.

In order to amplify across the gaps between the 10 S. enterica serovar Dublin contigs, PCR primers were designed to anneal near both ends of these contig sequences (see Table S1 in the supplemental material). PCR fragments were amplified from S. enterica serovar Dublin SD3246 genomic DNA by using the appropriate primer pairs, which showed that sequences similar to these contigs are present in SD3246 and that they link to form a large island. The complete DNA sequence of this S. enterica serovar Dublin SD3246 region was determined. However, as described above, only one boundary of the S. enterica serovar Dublin-specific island had been located. The required flanking region was obtained by using lambda red mutagenesis to specifically insert a mini-Tn5KmR transposon near the end of the island (Materials and Methods) and then cloning restriction fragments conferring kanamycin resistance from this mutant. In this way, a further ca. 6-kb region was cloned and its sequence was determined. The nucleotide sequence of most of this was highly homologous to those of several Salmonella serovars.

In total, a sequence of 26,210 bp was determined. BLASTN analysis of this sequence showed that only nucleotides 1 to 4561 and 25729 to 26210 are present in other sequenced salmonellae, with the central region of approximately 21 kb potentially being specific to S. enterica serovar Dublin. This novel region was designated SDI-1. SDI-1 has a G+C content of 51.3%, which is very similar to that of the S. enterica serovar Dublin genome. Since this work, the S. enterica serovar Dublin CT02021853 genome sequencing project has progressed, and the small contigs available then have now been assembled into larger contigs. Analysis of the updated genome sequence data shows that S. enterica serovar Dublin CT02021853 contains a corresponding island on contig ABAP01000038. There are only two nucleotide differences between the 26,210-bp regions of the two strains, with just one of these being within SDI-1.

The coding sequences of the 26,210-bp sequence were predicted using GLIMMER (Fig. 1 and Table 2). All the predicted ORFs are on the same strand. BLASTP and InterProScan were used to analyze the potential roles and functional domains of these proteins (Table 2). Products of ORF1 to ORF8, ORF10, ORF11, and part of ORF32 were predicted to be prophage proteins, while the ORF9 protein had no significant homologues. The other ORF proteins were most similar to Enterobacter sp. strain 638 proteins with unknown functions, except for Ent638_1033, which is predicted to be a NUDIX hydrolase. The lengths of the ORFs corresponded closely but not always exactly between S. enterica serovar Dublin and Enterobacter. The Enterobacter protein Ent638_1049 is predicted to contain a ubiquitin-activating enzyme 1 (E1) domain. Inter-
Interestingly this domain appears to be deleted from the corresponding *S. enterica* serovar Dublin ORF (ORF30). *S. enterica* serovar Dublin ORF27 has an N-terminal proline-rich 11-residue insertion which is not in Ent638_1046.

The addition of mitomycin C to cultures of *S. enterica* serovar Dublin SD3246 did not lead to phage induction under conditions that induced *S. enterica* serovar Typhimurium 4/74 phage and led to bacterial lysis (data not shown). No mitomycin C-induced bacterial lysis was detected for any of the *S. enterica* serovar Dublin isolates described in this study, and no phage DNA could be purified from mitomycin C-treated *S. enterica* serovar Dublin cultures under conditions that produced phage DNA from *S. enterica* serovar Typhimurium 4/74. Therefore, there is no evidence that SDI-1 is contained within an inducible prophage.

**Distribution of SDI-1 among *S. enterica* serovars.** Since SDI-1 was absent from all sequenced strains of *Salmonella*, its distribution among a larger set of strains was investigated. Genomic DNA was prepared from *S. enterica* serovar Dublin SD3246, *S. enterica* serovar Gallinarum SG9 and from 71 other *Salmonella* strains. These 71 isolates included 14 *S. enterica* serovar Dublin isolates and 30 other serovars (Materials and Methods). The DNA was used as a template in PCRs with three pairs of primers which amplify regions B, D, and F (Fig. 1 and Table 3). To confirm the presence or absence of SDI-1 in these 73 isolates, Southern blot analyses were performed. Two probes were prepared (P1 and P2) (Fig. 1) and hybridized to HindIII-digested genomic DNA. All the isolates that were positive by PCR and/or Southern blot are indicated by dark and light gray horizontal bars, respectively.

The results showed that all 15 isolates of *S. enterica* serovar Dublin have the island. Interestingly, two non-serovar Dublin isolates (*S. enterica* serovar Brandenburg and *S. enterica* serovar Duisburg) were positive in all 3 PCRs and on both Southern blots, suggesting they could possess the entire island. One isolate of *S. enterica* serovar Choleraesuis variant Decatur was also positive in four of the five tests. In addition, *S. enterica* serovar Heidelberg SARB24, *S. enterica* serovar Miami SARB29, and *S. enterica* serovar Murenchen SARB34 hybridized to one probe, suggesting that they might carry part of the island. Further PCRs were carried out on these six non-serovar Dublin isolates to determine the extent of the sequence similarity (Fig. 1 and Table 3). *S. enterica* serovar Duisburg was negative for PCR A only. Since one of the primers used in this PCR was outside the island, it is possible that the entire island is present but that the sequence adjacent to one end of it is different from that in *S. enterica* serovar Dublin strains. *S. enterica* serovar Brandenburg possessed much of the island, but the PCRs overlapping both ends of the island were negative, suggesting the possibility that it has inserted at a different location. The other serovars have smaller portions of the island.

**Mutagenesis of sequences identified by subtractive hybridization.** Transposon insertion mutants of 17 of the sequences identified in the subtraction library were generated in *S. enterica* serovar Dublin SD3246 Nal' as described earlier (Materials and Methods) to investigate the role of these sequences in vivo. The mutants each contained unique signature tags so that they could be tracked in complex pools during infection of calves as described previously (33). The sequences mutated in this way included most of the clones not similar to mobile elements (D1 to D17) except D5 and D8. The D5 sequence was not mutated, since it is part of the same gene as D4, and the D8 sequence was deleted in the mutant with the deletion of STM3021 to STM3030 (see below). In addition, the two sequences similar to RHS elements (D18 and D19) were mutated.

Seven additional tagged mutants were prepared as follows. The sequence of clone D7 was highly homologous to the *S. enterica* serovar Typhimurium LT2 gene STM3025, and D8 was homologous to STM3022. Comparisons of LT2 with the sequenced *S. enterica* serovar Gallinarum strain 287/91 showed that *S. enterica* serovar Gallinarum lacked a region of about 11 kb, containing STM3021 to STM3030. This region includes the stdABC fimbrial operon. A stdA mutant of strain SD3246 was therefore constructed, as well as a deletion mutant lacking the whole 11 kb. Another tagged deletion mutant, lacking the SDI-1 genes showing similarity to *Enterobacter* described above (from nucleotides 6076 to 25469, encoding ORF11 to ORF32 proteins), was constructed. This mutant was designated SD3246ΔSDI-1.

The subtractive hybridization sequences similar to those encoding phage-related proteins were not all individually mutated, since many of them encoded phage structural proteins that were considered unlikely to play a direct role in virulence. Several clones contained phage regions with similarity to *S. enterica* serovar Typhimurium prophages, including ST64B and Fels-2. These phages are absent from *S. enterica* serovar Gallinarum (30, 46), but large regions of both are present in the partially sequenced *S. enterica* serovar Dublin strain. PCR analysis confirmed these regions were also present in *S. enterica* serovar Dublin SD3246 (data not shown), and as noted above, there was evidence for a Fels-2-like phage carrying *S. enterica* serovar Dublin-specific genes. Two deletion mutants

![Fig. 1](https://example.com/f1.png)
### Table 2. Analysis of predicted ORFs on the *S. enterica* serovar Dublin SD3246 26,210-bp sequence containing SDI-1

<table>
<thead>
<tr>
<th>ORF</th>
<th>Nucleotides</th>
<th>G+C content (%)</th>
<th>Protein length (aa)</th>
<th>InterProScan domain(s)</th>
<th>Best BLASTP hit(s)</th>
<th>Homologue accession no.</th>
<th>Homologue length (aa)</th>
<th>% Identity (range of aa positions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Start–273</td>
<td>44.2</td>
<td>&gt;91</td>
<td></td>
<td>Bacteriophage protein STY1033 (<em>S. enterica</em> serovar Typhi CT18)</td>
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<td>101</td>
<td>100 (11–101)</td>
</tr>
<tr>
<td>2</td>
<td>316–918</td>
<td>52.9</td>
<td>200</td>
<td></td>
<td>Prophage proteins (<em>S. enterica</em> serovar Typhimurium LT2 Gifsy-1 STM2620 and <em>S. enterica</em> serovar Typhi CT18 STY1034)</td>
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<tr>
<td>3</td>
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<td>66</td>
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</tr>
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<td>4</td>
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<td>Phage lambda NinG</td>
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<td>Antitermination protein</td>
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<td>3417–4031</td>
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<td>204</td>
<td></td>
<td>Chitinase</td>
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<td>204</td>
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<td>183</td>
<td>Signal peptide, transmembrane domain</td>
<td></td>
<td></td>
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<td>94.7 (11–180)</td>
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<td>10</td>
<td>5044–6018</td>
<td>51.9</td>
<td>324</td>
<td>Terminus small subunit</td>
<td>Putative phage terminase small subunit (<em>Klebsiella pneumoniae</em>)</td>
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<td>334</td>
<td>62.7 (1–322)</td>
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<td>11</td>
<td>6008–7279</td>
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<td>NUDIX hydrolase</td>
<td>NUDIX hydrolase Ent638 1033 (<em>Enterobacter</em> sp. strain 638)</td>
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<td>54 (all)</td>
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<td>13636–14061</td>
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<td>141</td>
<td>Signal peptide</td>
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<td>YP_001175770</td>
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<td>50 (all)</td>
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<td>19</td>
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<td>148</td>
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<td>312</td>
<td></td>
<td>Hypothetical protein Ent638 1039 (<em>Enterobacter</em> sp. strain 638)</td>
<td>YP_001175772</td>
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<td>82.1 (all)</td>
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<td>145</td>
<td></td>
<td>Hypothetical protein Ent638 1041 (<em>Enterobacter</em> sp. strain 638)</td>
<td>YP_001175774</td>
<td>145</td>
<td>79.3 (all)</td>
</tr>
</tbody>
</table>

Continued on following page
of the Fels-2-like phage that lacked genes STM2694 to STM2706 and STM2694 to STM2722 were generated. Bacteriophage ST64B carries the effector gene \textit{sseK3}. An \textit{S. enterica} serovar Dublin \textit{sseK3} (sb26) mutant, as well as a deletion mutant lacking a large region of this phage (genes sb1 to sb25), was constructed.

Analysis of the role of \textit{S. enterica} serovar Dublin SD3246 genes identified by subtractive hybridization in calves. A pool containing the 24 mutants described above together with 12 control strains was inoculated orally into two calves (Materials and Methods). The controls consisted of three tagged virulent \textit{S. enterica} serovar Dublin SD3246 strains, three tagged virulent \textit{S. enterica} serovar Gallinarum SG9 strains, three \textit{S. enterica} serovar Dublin SD3246 type III secretion system-1 (T3SS-1) mutants, and three \textit{S. enterica} serovar Dublin SD3246 T3SS-2 mutants. The fate of these 12 control strains had previously

\begin{table}[h]
\centering
\caption{Distribution of SDI-1 among \textit{Salmonella} serovars}
\begin{tabular}{lccccccccc}
\hline
Strain/serovar (n) & A & B & C & D & E & F & G & P1 & P2 \\
\hline
\textit{S. enterica} serovar Dublin (15) & ND & + & ND & + & ND & + & ND & + & + \\
\textit{S. enterica} serovar Brandenburg S8 & – & + & + & + & + & – & + & + & + \\
\textit{S. enterica} serovar Duisburg S18 & – & – & + & + & + & – & + & + & + \\
\hline
\end{tabular}
\end{table}

\textit{a} The PCRs amplified regions as follows (see Fig. 1): A, bases 4532 to 5804; B, 7205 to 8312; C, 9887 to 11386; D, 15483 to 16857; E, 18148 to 20265; F, 21758 to 22573; G, 24017 to 25736. ND, not determined.

\textit{b} The probes were as follows (see Fig. 1): P1, bases 13820 to 14719; P2, bases 21758 to 22573.
been assessed in this model and they therefore serve as internal standards (33). Calves were anesthetized 72 h after oral inoculation, and jugular blood and efferent lymph were collected from a cannulated vessel draining the distal ileal loop as described in Materials and Methods. Biopsy specimens from distal ileal mucosa, draining MLN, liver, and spleen were collected at the end of each experiment. Duplicate dot blot hybridizations were performed with [32P]dCTP-labeled tags amplified from bacteria in the input and output pools from each site. Only one of the calves had bacteremia as detected by direct plating of blood. Representative blots obtained from this calf showing the fate of mutants at each site are shown in Fig. 2. The other calf gave comparable results in all the output pools except blood, of which a representative pool could not be obtained.

The three virulent S. enterica serovar Dublin SD3246 controls were present in efferent lymph and all enteric and systemic tissues examined 72 h after oral inoculation. In contrast, the serovar Gallinarum SG9 controls had been cleared from all sites by this time. The T3SS-1 and T3SS-2 apparatus mutants were also recovered in smaller quantities than the input amounts at enteric sites by 3 days postinoculation and were not recovered from systemic sites or lymph. None of the other mutants appeared to be underrepresented in any of the output pools compared to the input, suggesting that their ability to invade, translocate, or persist in enteric or systemic tissues was not substantially reduced.

**Functional characterization of SDI-1.** To further evaluate the contribution of SDI-1 to the virulence of S. enterica serovar Dublin in vivo, the phenotype of SD3246ΔSDI-1 relative to the parent strain was assessed in calves in competition experiments. Six calves were orally inoculated with a mixture of equal numbers of S. enterica serovar Dublin SD3246 wild type (Nalr) and SD3246ΔSDI-1 organisms. The CIs were determined at enteric and systemic sites 3 days postinfection as described in Materials and Methods (Table 4). The CIs were consistently below 1, with mean values ranging from 0.631 to 0.792 for the tissues examined. A Mann-Whitney nonparametric test indicated that the output ratios for all sites were significantly lower than the input ratio in the inocula (the *P* value was 0.0048 at all sites; Table 4). In contrast, the in vitro CI for this mutant in minimal medium was 1.12. Although the level of attenuation was modest, these results indicate that SDI-1 contributes to the pathogenicity of S. enterica serovar Dublin in calves.

The role of SDI-1 was next investigated using cultured-cell assays. In J774 murine macrophage-like cells, the SD3246ΔSDI-1 mutant was killed at a rate similar to the rate at which the wild type was killed (Fig. 3A). An S. enterica serovar Dublin ΔphoPQ mutant created by linear recombination was killed at a significantly higher rate than the wild type was, as expected (*P* = 0.001). The SD3246ΔSDI-1 mutant also entered INT407

**FIG. 2.** Analysis of the role of S. enterica serovar Dublin (SD) SD3246 genes absent from S. enterica serovar Gallinarum (SG) SG9 in invasion of distal ileal mucosa, spread to draining MLN, lymphatic translocation, and dissemination to organs and blood. Representative blots from one calf show the prevalence of defined signature-tagged SD3246 mutants from tissues, blood, and lymph at 72 h post-oral inoculation relative to the input. Row A contains the controls as follows: wells A1 to A3, virulent tagged S. enterica serovar Dublin SD3246 T3SS-2 mutants; A4 to A6, virulent tagged S. enterica serovar Dublin 3246 controls; A7 to A9, S. enterica serovar Dublin SD3246 T3SS-1 mutants; and A10 to A12, S. enterica serovar Dublin SD3246 T3SS-2 mutants. Rows B and C show S. enterica serovar Dublin SD3246 mutants prepared in this study as follows: well B1, clone D1; B2, clone D2; B3, clone D3; B4, clone D4; B5, clone D6; B6, clone D7; B7, clone D9; B8, clone D10; B9, clone D11; B10, clone D12; B11, clone D13; B12, clone D14; C1, clone D15; C2, clone D16; C3, clone D17; C4, clone D18; C5, clone D19; C6, SD3246ΔSDI-1; C7, STM3021 to STM3030 deletion mutant; C8, std4 deletion mutant; C9, sb1 to sb25 deletion mutant; C10, seSK3 deletion mutant; C11, STM2694 to STM2706 deletion mutant; and C12, STM2694 to STM2722 deletion mutant.

**TABLE 4.** Competitive indices for SD3246ΔSDI-1 in calves

<table>
<thead>
<tr>
<th>Site</th>
<th>CI for indicated calf</th>
<th>Mean CI (SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileal mucosa</td>
<td>0.556 0.721 0.702 0.778 0.497 0.769</td>
<td>0.671 (0.048)</td>
</tr>
<tr>
<td>MLN</td>
<td>0.663 0.822 0.904 0.794 0.785 0.786</td>
<td>0.792 (0.032)</td>
</tr>
<tr>
<td>Lymph</td>
<td>0.593 0.635 0.803 0.727 0.682 0.706</td>
<td>0.691 (0.030)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.643 0.444 0.589 0.749 0.788 0.763</td>
<td>0.663 (0.054)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.434 0.581 0.724 0.725 0.603 0.718</td>
<td>0.631 (0.047)</td>
</tr>
</tbody>
</table>

*The Mann-Whitney test was used to determine whether the output ratio was significantly different from the input ratio at each of the five sites. A *P* value of 0.0048 was obtained at all sites.
The majority of mice, the CI was mice of this type with SD3246 at this dose. Nevertheless, for a bottleneck in the establishment of persistent infection in 6 days postinoculation) were more variable, possibly owing to remaining mice that presented disease at later time points (up to 0.12 in the spleen and liver, respectively. The CIs in the re-

follows:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>●</td>
<td>Wild-type NaI strain; SD3246ΔSDI-1; and pshOQ mutant.</td>
</tr>
<tr>
<td>■</td>
<td>Wild-type NaI strain; SD3246ΔSDI-1; and sipD mutant.</td>
</tr>
<tr>
<td>◆</td>
<td>Wild-type NaI strain; SD3246ΔSDI-1; and phoPQ mutant.</td>
</tr>
<tr>
<td>▲</td>
<td>Wild-type NaI strain; SD3246ΔSDI-1; and phoPQ mutant.</td>
</tr>
</tbody>
</table>

Data points represent the means ± standard errors of three or four independent experiments, with triplicate wells.

Intestinal epithelial cells in numbers similar to those of the parent strain (1-h time point; Fig. 3B) and intracellular replication was comparable at 24 h. As expected, an S. enterica serovar Dublin SPI-1 (sipD) mutant was substantially impaired in its ability to invade INT407 cells. By 3 days postinfection, SD3246ΔSDI-1 was recovered in numbers that were signifi-

antly higher than those of the wild type (P = 0.014). Cytotox-

icity induced by these two strains was not significantly different as determined by a lactate dehydrogenase release assay (data not shown). Taken together with the CI during growth in minimal media, these data indicate that the SDI-1 mutation does not exert a fitness cost per se.

To investigate whether SDI-1 is a host-specific virulence factor, we also performed competition experiments with inbred mice. A dose of ca. 2 × 10^6 CFU comprising equal amounts of SD3246ΔSDI-1 and F. phoPQ mutant; SD3246ΔSDI-1; and sipD mu-

tant. Data points represent the means ± standard errors of three or four independent experiments, with triplicate wells.

FIG. 3. Interaction of S. enterica serovar Dublin SD3246 wild-type and mutant strains with cultured cells. (A) Survival in J774 cells. The symbols for strains are as follows: ●, wild-type NaI strain; ▲, SD3246ΔSDI-1; and ◆, phoPQ mutant. (B) Invasion, intracellular growth, and survival in INT407 cells. The symbols for strains are as follows: ●, wild-type NaI strain; ■, SD3246ΔSDI-1; and ▲, sipD mu-

tant. Data points represent the means ± standard errors of three or four independent experiments, with triplicate wells.

intestinal epithelial cells in numbers similar to those of the parent strain (1-h time point; Fig. 3B) and intracellular replication was comparable at 24 h. As expected, an S. enterica serovar Dublin SPI-1 (sipD) mutant was substantially impaired in its ability to invade INT407 cells. By 3 days postinfection, SD3246ΔSDI-1 was recovered in numbers that were signifi-

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icity induced by these two strains was not significantly different as determined by a lactate dehydrogenase release assay (data not shown). Taken together with the CI during growth in minimal media, these data indicate that the SDI-1 mutation does not exert a fitness cost per se.

To investigate whether SDI-1 is a host-specific virulence factor, we also performed competition experiments with inbred mice. A dose of ca. 2 × 10^6 CFU comprising equal amounts of SD3246ΔSDI-1 and the parent strain was given to 16 female C57BL/6 mice by oral gavage. Animals were humanely killed upon presentation of symptoms of salmonellosis, and homog-

enates of spleen and liver were plated for the determination of CIs. Bacteria were recovered from both sites in all mice (at least 7 × 10^5 CFU). Four mice that presented disease at 3 to 4 days postinoculation had mean CIs of 1.01 ± 0.07 and 1.17 ± 0.12 in the spleen and liver, respectively. The CIs in the re-

maining mice that presented disease at later time points (up to 6 days postinoculation) were more variable, possibly owing to a bottleneck in the establishment of persistent infection in mice of this type with SD3246 at this dose. Nevertheless, for the majority of mice, the CI was >1 (9 of 12 spleen samples and 8 of 12 liver samples), with 6 of the 12 mice yielding only the mutant strain at post mortem examination. Taken to-

gogether, these data indicate that SDI-1 is not required for vir-

ulence in mice and imply that it may play a host-specific role in S. enterica serovar Dublin pathogenesis in cattle.

**DISCUSSION**

The bacterial and host factors that determine why some S. enterica serovars translocate to distal sites while others are restricted to the gastrointestinal tract are ill defined. We pre-

viously showed that the ability of S. enterica serovar Dublin to persist in bovine ileal mucosa and translocate via efferent lymphatics compared with other serovars in cattle correlated with systemic virulence (28, 33). In contrast, the systemic virulence of host-restricted serovars did not correlate with intramacrophage survival (44) or with invasion or damage of the ileal mucosa (9, 27, 28, 42) but did correlate with reduced net replication in the intestinal wall and with reduced inflammation in the ileum (27). It has been shown that S. enterica serovar Typhi reduces Toll-like receptor-dependent interleukin-8 expression and subsequent inflammation in the intestinal mucosa by a process requiring the Vi capsular antigen (34). These findings suggest that the greater induction of proinflammatory re-

sponses by rapidly proliferating ubiquitous serovars might result in them being confined to the intestines, whereas host-

restricted and host-specific serovars may have developed mechanisms to evade or suppress activation of host innate immunity at mucosal surfaces and thus disseminate to distal sites.

The screening of mutant banks of the ubiquitous serovar S. enterica serovar Typhimurium has shown that different genes are utilized to colonize different animal hosts (8, 25, 40). The repertoire and sequence of such factors have the potential to influence host and tissue tropism. For example, it has been established that different serovars express different sets of fimbrial operons (reviewed in reference 13) and that adaptation to the avian host is often associated with the loss of type 1 fimbriae and motility (10, 23). It has also been suggested that factors involved in host restriction may have a metabolic basis. For example, S. enterica serovar Dublin is a nicotinic acid auxotroph, and it is interesting that cattle can synthesize nicotinic acid and do not require niacin in their diet (16).

In this study, we have identified genes present in S. enterica serovar Dublin SD3246 but not S. enterica serovar Gallinarum SG9 to dissect the genetic basis of the differential virulence of these two strains in cattle. It is difficult to estimate the per-

centage of S. enterica serovar Dublin-specific sequences that we have identified here, due to the absence of a complete S. enterica serovar Dublin genome sequence. Our library identi-

fied three fragments totaling approximately 2 kb on the 21-kb SDI-1. If this is representative, it suggests coverage of just under 10%. We identified a total of 41 S. enterica serovar Dublin SD3246 DNA sequences that were absent from S. enterica serovar Gallinarum SG9. As expected, many of these corresponded to mobile elements, particularly prophage genes. Another group was present in a range of serovars, including ubiquitous serovars, and some of the differences had previously been reported (30). For example, S. enterica serovar Dublin SD3246 fragments D1 and D2 (Table 1) corresponded to the Salmonella microarray region B16, SD3246 fragment D13 was
on microarray region A02, and fragment D6 was on microarray region B08 (30). These regions had been shown, by use of the microarray, to be present in S. enterica serovar Dublin but absent from some S. enterica serovar Gallinarum isolates (30), which is consistent with our findings.

Of particular interest were the sequences that were unique to S. enterica serovar Dublin. Further analysis of these showed that two of the unique sequences, D14 and D15, were on a Fels-1-like prophage, and two others, D16 and D18, were associated with RHS genetic elements. The roles of these four regions are unknown, but these fragments all had very low G+C contents, indicating that they may have been acquired relatively recently by lateral gene transfer. Interestingly, a number of Salmonella virulence factors located on prophages have previously been described; for example, S. enterica serovar Typhimurium LT2 has four prophages that all carry one or more genes involved in virulence, such as nanH and sodCIII on the Fels-1 prophage (14). Also, the horizontally acquired SPI-6, which potentially carries a T6SS and the saf fimbrial operon, contains an RHS element (17, 31).

Comparisons with the databases suggested that D11 and D12 might also be specific to S. enterica serovar Dublin. Analysis of the flanking regions showed that these were carried on a 21-kb genomic island designated SDI-1. This island was present in all S. enterica serovar Dublin isolates studied, and its sequence was very highly conserved between isolates SD3246 and CT02021853. Such high sequence conservation between different isolates of host-restricted serovars, such as S. enterica serovar Dublin, has been noted previously (36). SDI-1 was absent from most other serovars. Exceptions included single isolates of S. enterica serovar Duisburg and S. enterica serovar Brandenburg. Analysis of the SDI-1 sequence gave few clues about the potential function of genes on the island. Although SDI-1 was flanked by phage sequences, S. enterica serovar Dublin isolates did not contain inducible prophages, suggesting that insertion of the island into a prophage may have disrupted the phage. SDI-1 ORF11 to ORF32 proteins were similar at the amino acid level to predicted proteins of Enterobacter sp. strain 638, an endophytic strain which was isolated from a plant. However, the level of nucleotide similarity was very low, so the island is unlikely to have recently originated from Enterobacter. The evolutionary origin of SDI-1 remains unknown.

Other regions which might be involved in the virulence or host restriction of S. enterica serovar Dublin were those that had very limited distributions among serovars, such as D4 and D5, which were found only in S. enterica serovar Dublin and S. enterica serovar Paratyphi A. The translated D4 and D5 sequences were highly similar to the N terminus of the S. enterica serovar Paratyphi A gene product, SPA2350. Although the N-terminal region of this protein is unusual, the C terminus is highly conserved in a number of S. enterica and E. coli proteins that are predicted to be autotransporters and/or virulence factors.

Screening of a pool of defined signature-tagged mutants with insertions in the subtractive hybridization library sequences in a calf model did not identify any attenuated mutants. The method confirmed attenuation of SG9 and SD3246 tagged SPI-1 and SPI-2 mutant strains detected previously (33). However, subtle attenuating effects could not be ruled out. Indeed, a competition experiment comparing the SDI-1 deletion mutant with the parent wild-type strain showed that this mutant was outcompeted by the wild type at all sites tested. This suggests the mutant colonized or persisted less well than the wild type in vivo. The CIs obtained were between 0.434 and 0.904, showing that the attenuation was less than that previously observed for T3SS-1 mutants in calves (which had CIs below 0.1 in efferent lymph and MLN 12 h after instillation into ligated ileal loops) (33). However, the CIs were consistent and the attenuation was statistically significant. Since it is likely that a number of genes are required for host adaptation (40), it is perhaps not surprising that the inactivation of one region caused such modest attenuation. No defects in invasion of cultured epithelial cells or intramacrophage survival could be detected for the SDI-1 mutant relative to the parent in assays that confirmed the known attenuating effect of SPI-1 or PhoPQ mutation. Taken together with the CI during growth in minimal medium, these data imply that SDI-1 mutation does not compromise fitness. In a murine model, when signs of systemic salmonellosis first appeared, mean CIs in the spleen and liver exceeded 1. While at later time points CIs were more variable, the mutant strain predominantly outcompeted the wild type, indicating that SDI-1 is not required for virulence in mice and may play a host-specific role in cattle.

This is the first report of an S. enterica serovar Dublin-specific locus that contributes to virulence in the bovine host. Further studies will be required to determine whether any of the other S. enterica serovar Dublin-specific regions identified here play subtle roles in host adaptation and virulence. It is also likely that other genetic mechanisms not examined here, such as gene deletions, differential expression of orthologous genes, or allelic differences in orthologous sequences, contribute to the systemic virulence of S. enterica serovar Dublin. However, the finding that SDI-1 plays a role in S. enterica serovar Dublin virulence implies that host restriction (and more severe disease outcomes) may not be solely due to gene decay but may require the acquisition of specific factors.

ACKNOWLEDGMENTS

This work was supported by the Biotechnology and Biological Sciences Research Council and the Department for the Environment, Food and Rural Affairs (grant CS0964X).

We thank D. Prickett and M. Watson for bioinformatics support and D. Hudson and G. Prescott for technical assistance.

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