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Identification by Subtractive Hybridization of Sequences Specific for *Salmonella enterica* Serovar Enteritidis

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Salmonella enterica **serovar Enteritidis, a major cause of food poisoning, can be transmitted to humans through intact chicken eggs when the contents have not been thoroughly cooked. Infection in chickens is asymptomatic; therefore, simple, sensitive, and specific detection methods are crucial for efforts to limit human exposure. Suppression subtractive hybridization was used to isolate DNA restriction fragments present in *Salmonella* serovar Enteritidis but absent in other bacteria found in poultry environments. Oligonucleotide primers to candidate regions were used in polymerase chain reactions to test 73 non-Enteritidis *S. enterica* isolates comprising 34 different serovars, including Dublin and Pullorum, two very close relatives of Enteritidis. A primer pair to one *Salmonella* difference fragment (termed Sdf I) clearly distinguished serovar Enteritidis from all other serovars tested, while two other primer pairs only identified a few non-Enteritidis strains. These primer pairs were also useful for the detection of a diverse collection of clinical and environmental *Salmonella* serovar Enteritidis isolates. In addition, five bacterial genera commonly found with *Salmonella* serovar Enteritidis were not detected. By treating total DNA with an exonuclease that degrades sheared chromosomal DNA but not intact circular plasmid DNA, it was shown that Sdf I is located on the chromosome. The Sdf I primers were used to screen a *Salmonella* serovar Enteritidis genomic library and a unique 4,060-bp region was defined. These results provide a basis for developing a rapid, sensitive, and highly specific detection system for *Salmonella* serovar Enteritidis and provide sequence information that may be relevant to the unique characteristics of this serovar.**

In the last few decades, *Salmonella enterica* serovar Enteritidis has emerged as a major cause of food-borne illness worldwide. This pathogen is distinguished from its many close relatives also found in poultry environments by its ability to infect chicken ovaries before the eggshell is formed, allowing transmission through intact eggs. Once established in the human host from raw or undercooked eggs or egg products, this bacterium causes gastroenteritis similar to other *S. enterica* serovars. Infection in poultry flocks, which is asymptomatic, was first noticed in the late 1970s and in the 1980s spread rapidly throughout the United Kingdom, the United States, South America, and other areas. During this period, the proportion of salmonellosis cases attributed to *Salmonella* serovar Enteritidis increased substantially, showing a 275-fold increase in Argentina and becoming the predominant cause of this disease in the United States (10, 11, 14). Baumler et al. suggested that this rapid increase of *Salmonella* serovar Enteritidis may have been due to successful campaigns to eradicate the *Salmonella* serovars Pullorum and Gallinarum, the causative agents in chickens of bacillary white diarrhea and fowl typhoid, respectively (2). It is hypothesized that these avian-adapted salmonellae provided cross-immunity against *Salmonella* serovar Enteritidis because of important similarities in lipopolysaccharide structures. Therefore, these campaigns may have opened an ecological niche that has since been occupied by *Salmonella* serovar Enteritidis. This view remains controversial, however, since serovars Gallinarum and Pullorum remain prevalent in many developing countries where serovar Enteritidis has nevertheless increased dramatically, and turkey flocks in developed countries, now free of serovars Gallinarum and Pullorum, have not been colonized by serovar Enteritidis (12, 15). Unlike the avian-adapted salmonellae, rodents serve as an animal reservoir for *Salmonella* serovar Enteritidis, suggesting that culling would not be an effective method of control. It is possible that the use of *Salmonella* serovar Enteritidis as a rodenticide may have contributed to the current prevalence of this serovar, and it is also likely that infected rodents are currently a source of disease. In addition to the health risks, this pathogen has had a significant economic impact on the egg industry through decreased consumer confidence following well-publicized outbreaks.

Although *Salmonella* serovar Enteritidis is closely related to other pathogenic *S. enterica* serovars, several characteristics of this serovar appear to distinguish it from many others. For example, the fimbria Sef14 is found in a limited number of *S. enterica* serovars, including Enteritidis. This surface structure appears to be required for macrophage uptake and survival in intraperitoneal infections (6) in contrast to other *Salmonella* fimbriae that promote binding to host epithelial cells (5). There is also evidence that quorum sensing plays an important role in the life cycle of *Salmonella* serovar Enteritidis. Viru-

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lence correlates with a strain's ability to produce high-molecular-weight lipopolysaccharide and the ability of subpopulations to grow to high cell densities (8).

Because of the increased prevalence of *Salmonella* serovar Enteritidis and its complex life cycle, rapid and effective detection methods are important as a basis of control. Traditional culture methods require several days. More rapid methods have been developed but are often based on the *sef* operon, which is also found in other serogroup D salmonellae. One such test relies on recombinant Sef14 antigen to detect *Salmonella* serovar Enteritidis antibodies in chickens, but this test requires that serum samples be collected and also detects antibodies against *S. enterica* serovar Dublin (13). The gene encoding Sef14, *sefA*, is also found in the serovars Blegdam, Gallinarum, Pullorum, Rostock, Seremban, and Typhi (20). Several PCR-based assays have also been reported. One is based on *sefA* (18), and another is based on plasmid-borne sequences (17, 23). While the latter test appears quite specific for *Salmonella* serovar Enteritidis, the diversity of the isolates used for this study, normally based on phage typing, is unclear. Furthermore, since plasmids are often mobilizable and unstable, a plasmid-based test might not detect the occurrence of plasmid-less strains that could rapidly acquire virulence by plasmid transfer. Rapid plasmid transfer to plasmid-less strains is an important aspect of virulence in the plant pathogen *Agrobacterium tumefaciens* and may be important in other pathogens (7, 21, 24).

Here we describe the identification of a novel *S. enterica* serovar Enteritidis locus that serves as a marker for DNA-based identification of this bacterium. In contrast to other tests, this marker is not found in a wide range of closely related serovars, including Dublin and Pullorum, the two closest relatives of Enteritidis (19). Thus, this test allows highly specific detection of *Salmonella* serovar Enteritidis. Evidence is presented supporting a chromosomal location for the locus, thus circumventing the potential problems associated with plasmid-borne markers. An extensive array of *Salmonella* serovar Enteritidis phage types from around the world was tested by PCR for the presence of this DNA region, and all phage types associated with human infections were detected. An ~7-kb region was isolated by PCR-based screening of a *Salmonella* serovar Enteritidis library and subsequently sequenced. The region of this clone that does not match the *S. enterica* serovar Typhi or Paratyphi complete genomes contains six short open reading frames (ORFs). The putative proteins show either weak or no similarity to database sequences. Two other primer pairs were developed that are also quite effective at detecting *Salmonella* serovar Enteritidis. The combined use of these primer pairs provides tools for developing rapid and specific detection methods for *S. enterica* serovar Enteritidis.

MATERIALS AND METHODS

Strains. Strains used for these studies are listed in Tables 1, 2, and 3. Some strains, as indicated in the text, were obtained from the American Type Culture Collection (ATCC), Rockville, Md. Serotyping was verified or performed by the California Animal Health and Food Safety Laboratory (CAHFS) by using standard procedures. The National Veterinary Services Laboratory (NVSL), Ames, Iowa, performed phage typing by standard methods (9).

DNA preparation. DNA was isolated from 3-ml cultures after overnight growth in Luria-Bertani medium (Sigma, St. Louis, Mo.). Either of two methods was used to purify total DNA; both methods yielded consistent results. DNA STAT-60 isolation reagent (Tel-Test, Friendswood, Tex.) was used according to the manufacturer's recommendations (1 ml per culture). Alternatively, cell pellets were resuspended in 200 μl of TE buffer (10 mM Tris HCl, 1 mM EDTA; pH 8.0) and treated with 2.5 μg of proteinase K/ml for 30 min at 37°C. Successive extractions were performed with saturated phenol, phenol-chloroform, (1:1, vol/vol) and chloroform-isoamyl alcohol (24:1, vol/vol). DNA was precipitated with 0.5 ml of cold 95% ethanol and 75 μl of 3 M sodium acetate (pH 5.2), dried under vacuum in a desiccator, and resuspended in water.

DNA amplification for strain testing. Oligonucleotide primers (Sigma-Genosys, The Woodlands, Tex.) at a 400 nM final concentration were combined with 200 pg of genomic DNA template and amplified with Advantage 2 Polymerase (ClonTech, Palo Alto, Calif.). After an initial denaturation at 94°C for 1 min, the samples were subjected to 27 cycles of 94°C for 30s, 58°C for 30s, and 72°C for 1 min, followed by a final 7-min incubation at 72°C. Samples were fractionated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. A primer pair to either the 23S or 16S rRNA gene was used as a positive control for the amplification of each DNA sample, or a primer pair to the *rplI* gene (encoding the L9 ribosomal protein) was used as an internal control.

Suppression subtraction hybridization, DNA sequencing, and analysis. Genome comparisons by suppression subtraction hybridization were performed essentially as described by Akopyants et al. (1) with the following exceptions. For subtractions with *Sau*3AI-digested DNA, adapter 1 was formed by annealing the adapter 1 long oligonucleotide with the oligonucleotide 5′ GATCACCTGCC CGG to form an adapter with appropriate cohesive ends. Similarly, adapter 2 was formed by annealing the adapter 2 long oligonucleotide with the oligonucleotide 5′-GATCCAATCGGCCG. Ligase (New England Biolabs, Beverly, Mass.) was inactivated by incubation at 72°C for 20 min. Unpurified PCR products were cloned using the pGEM-T Easy TA cloning kit (Promega, Madison, Wis.). Recombinant clones were picked by using a BioRobotics (Woburn, Mass.) BioPick automated colony picker, and plasmid templates were prepared by boiling lysis and magnetic bead capture with a high-throughput procedure (16). Sequencing of plasmid templates was performed by using the Applied Biosystems (Foster City, Calif.) BigDye Terminator system and either ABI 377 or 3700 automated sequencers. The sequencing primers used were 5′-TGTAAAACGA CGGCCAGT (forward) and 5′-CAGGAAACAGCTATGACC (reverse). Sequences were assembled and oligonucleotide primers were designed by using the Consed software package (University of Washington, Seattle). Sequence comparisons with the GenBank databases were performed using the BLAST (basic local alignment search tool) server at the Baylor College of Medicine (Houston, Tex.) or the server at the National Center for Biotechnology Information (Bethesda, Md.). Both the nonredundant and the unfinished microbial databases were used for comparisons.

Oligonucleotide primers. The sequences of the primer pairs used (Sigma-Genosys) for DNA amplification were as follows: *spvC*, 5′-CTCTGCATTTCA CCACCATCACG and 5′ CTTGCACAACCAAATGCGGAAGAT; *rplI*, 5′ GGGTGATCAGGTTAACGTTAAAG and 5′CTTCGTGTTCGCCAGTGGT ACGC; 23S, 5′-CTACCTTAGGACCGTTATAGTTAC and 5′-GAAGGAACT AGGCAAAATGGTGCC; 16S, 5′AGAGTTTGATCCTGGCTCAG and 5′-GG TTACCTTGTTACGACTT; Sdf I, 5′-TGTGTTTTATCTGATGCAAGAGG and 5′-CGTTCTTCTGGTACTTACGATGAC; Sdf II, 5′-GCGAATATCATT CAGGATAAC and 5′-GCATGTCATACCGTTGTGGA; and Sdf III, 5′-GCT GACTCACACAGGAAATCG and 5′-TCTGATAAGACTGGGTTTCACT.

DNase assays. Plasmids were prepared from *Salmonella* serovar Enteritidis CAHFS-285, by a standard alkaline lysis method (4), except that proteins and cell debris were precipitated with 7.5 M ammonium acetate (1/2 volume) instead of sodium acetate. The DNA from a 10-ml culture was resuspended in 40 μl of TE, and 10 μl was digested with Plasmid-Safe DNase (Epicentre Technologies, Madison, Wis.) in a 250-μl reaction with 50 U of enzyme for 5 h according to the manufacturer's recommendations. Then, 5 μl of this reaction was used as a template in PCRs (30 cycles of 1 min of annealing at 65°C, 1 min of extension at 72°C, and 30 s of denaturation at 94°C).

Library construction and screening. To construct a genomic library of *Salmonella* serovar Enteritidis strain CAHFS-285, 100 μg of total DNA was partially digested with 100 U *Sau*3AI (New England Biolabs) for 10 min. The DNA was fractionated by electrophoresis, and 4- to 6-kb fragments were excised and gel purified by electroelution. These fragments were ligated to pUC9 (22) that had been digested with *Bam*HI (New England Biolabs), gel purified, and treated with shrimp alkaline phosphatase (U.S. Biochemicals, Cleveland, Ohio). Products were introduced into *Escherichia coli* DH10B cells (Gibco-BRL, Rockville, Md.) by electroporation (Gene-Pulser; Bio-Rad, Richmond, Calif.), and transformants were selected with 50 μg of ampicillin (Sigma, St. Louis, Mo.)/ml on agar plates with Luria-Bertani (LB) medium. By using a BioPick automated colony picker, white colonies (total of 6,528) were used to inoculate 384-well microtiter plates

(Nalge Nunc, Rochester, N.Y.) containing LB medium with 7.5% (vol/vol) glycerol, followed by overnight incubation at 37°C. The library was replicated with a 384-pin tool and stored at −70°C. Screening was performed with the Sdf I primers by amplification of combined cultures, followed by amplification of single cultures. For each row, 5 μl of each culture was combined, and 1 μl of the mixture was PCR tested. For rows with a positive signal, the individual clones were then tested. One clone consistently yielded positive results in PCRs and was selected for sequencing.

DNA sequencing of the Sdf I region. The library clone identified by PCR with the Sdf I primers was purified by alkaline lysis and anion-exchange chromatography with a Qiagen (Valencia, Calif.) Plasmid Preparation Kit. The plasmid DNA was digested with *Eco*RI and *Hin*dIII and separated by electrophoresis, and the two insert fragments were gel purified using a Qiaex II kit (Qiagen). The purified fragments were first treated with the Klenow fragment of DNA polymerase I (New England Biolabs) and deoxynucleoside triphosphates, followed by digestion with *Alu*I, *Hae*III, and *Rsa*I in separate reactions. Then, the products from each of the three reactions were separately cloned into pPA9 that had been digested with *Eco*RV and treated with shrimp alkaline phosphatase. The plasmid pPA9 was constructed by annealing the oligonucleotides 5′ AGCTTGGAATT CGATATCAGGCCTCG and 5′ GATCCGAGGCCTGATATCGAATTCCA, which were then cloned between the *Hin*dIII and *Bam*HI sites of pUC9 (22). We sequenced 32 clones from each enzyme sublibrary (96 total) as described above and assembled overlapping sequences with the Consed program to generate the complete sequence of the insert. The assembly was corroborated with restriction mapping based on the sequence.

Nucleotide sequence accession numbers. The sequences for *Salmonella* difference fragments (Sdf) I to IX from *S. enterica* serovar Enteritidis CAHFS-5 have been submitted to GenBank with accession numbers AF370707 to AF370715, respectively. The sequence for *Salmonella* difference region I (Sdr I) from *S. enterica* serovar Enteritidis CAHFS-285 has been submitted to GenBank with accession number AF370716.

RESULTS

Isolation of DNA fragments unique to *S. enterica* serovar Enteritidis. Suppression subtractive hybridization (SSH) was used to identify *Salmonella* serovar Enteritidis-specific sequences that could serve as diagnostic markers. SSH is a PCR-based technique that enriches for restriction fragments that are present in one strain, termed the tester, but absent in another, termed the driver. *Salmonella* serovar Enteritidis strain CAHFS-5 was used as the tester (phage type 8), and the closely related serovar Dublin (strain CAHFS-9008117D), also in serogroup D1, was used as the driver. This way, any true SSH products would be likely to distinguish serovar Enteritidis from serovar Dublin and its close relatives. Four restriction enzymes were used in separate SSH experiments: *Rsa*I, *Alu*I, *Sau*3AI, and *Hae*III. We sequenced 48 clones from each subtraction (192 total) and synthesized PCR primers for 98 of the products. Ninety-four clones with high similarity to available non-Enteritidis database sequences were not studied further.

PCR amplifications were then performed by using the driver and tester DNAs as templates to identify true subtraction products. Nine primer pairs showed amplification with *Salmonella* serovar Entertitidis but not with serovar Dublin. These unique restriction fragments from which the primers were designed were designated Sdf I to Sdf IX (*Salmonella* difference fragment). One of the nine fragments was from an SSH experiment using *Sau*3AI (Sdf I), one was an *Alu*I fragment, five were *Hae*III fragments (including Sdf II and Sdf III), and two were *Rsa*I fragments. The primer pairs (referred to as "Sdf I primer pair," etc.) based on these nine sequences were selected for further analysis.

Characterization of DNA fragments unique to *Salmonella* serovar Enteritidis. The nine primer pairs that amplified sequences from *Salmonella* serovar Enteritidis but not serovar Dublin were PCR tested with several other serovars commonly found in the poultry environment to eliminate those primer pairs that were not serovar Enteritidis specific. Amplification of sequences from one isolate each of *Salmonella* serovars Typhimurium, Heidelberg, Montevideo, and another isolate of *Salmonella* serovar Enteritidis (CAHFS-285, a phage type 4 strain) were used for this purpose. In addition, the two strains used in the subtraction (*Salmonella* serovar Dublin CAHFS-9008117D and *Salmonella* serovar Enteritidis CAHFS-5) were included as controls. Three of the nine primer pairs detected both strains of serovar Enteritidis but none of the other serovars. These three primer pairs were further evaluated using an extensive collection of *S. enterica* serovars available at the CAHFS. We also tested 81 additional *S. enterica* isolates, including 30 additional serovars (for a total of 34 non-Enteritidis serovars, including those described above [Table 1]) and 12 additional serovar Enteritidis environmental and poultry isolates (Table 2). Most of the 34 non-Enteritidis serovars are encountered at egg production facilities and therefore complicate diagnostic efforts to detect serovar Enteritidis. The Sdf II primer pair identified 7 of the 73 non-Enteritidis isolates, representing six non-Enteritidis serovars. Interestingly, one of the strains was an isolate of *Salmonella* serovar Dublin, even

TABLE 1. PCR results for selected *S. enterica* serovars

Serovar (no. of strains)	Serogroup	Region evaluated (no. of strains positive)		
		Sdf I	Sdf II	Sdf III
Agona (2)	B	0	2	0
Derby (2)	B	0	0	0[a]
Heidelberg (3)	B	0	0	0
Reading (1)	B	0	0	0[a]
Typhimurium (4)	B	0	0	0
Infantis (2)	C1	0	0	0
Lille (1)	C1	0	0	0
Livingstone (3)	C1	0	1	0
Mbandaka (2)	C1	0	0	0[a]
Montevideo (3)	C1	0	0	0
Ohio (2)	C1	0	0	0
Oranienburg (3)	C1	0	0	0
Tennessee (2)	C1	0	0	0
Thompson (1)	C1	0	0	0
Blockley (1)	C2	0	0	0[a]
Newport (2)	C2	0	0	0
Corvallis (1)	C3	0	0	0
Kentucky (4)	C3	0	0	0[a]
Berta (1)	D1	0	1	0[a]
Dublin (7)	D1	0	1	0
Lomalinda (2)	D1	0	0	0[a]
Panama (1)	D1	0	0	0
Pullorum (1)	D1	0	1	0
Fresno (1)	D2	0	0	0
Anatum (1)	E1	0	0	0
Give (2)	E1	0	0	0
Muenster (2)	E1	0	0	0
Cambridge (1)	E2	0	0	0
Newbrunswick (2)	E2	0	0	0
Newington (2)	E2	0	0	0
Menhaden (3)	E3	0	0	0
Senftenberg (2)	E4	0	0	0
Worthington (2)	G2	0	1	0
Cerro (4)	K	0	0	0

[a] Products other than the expected size detected in one or more strains of this serovar.

TABLE 2. PCR results for selected *S. enterica* serovar Enteritidis strains from various environmental sources

Isolate no.	Source	State, district, or country of origin	Phage type	Region evaluated[a]		
				Sdf I	Sdf II	Sdf III
D1850	Human, feces[b]	Texas	4	Pos	Pos	Pos
D0918	Human, feces[b]	Mexico	4	Pos	Pos	Pos
D1832	Human, feces[b]	Italy	7	Pos	Pos	Pos
D0119	Human, feces[b]	Spain	4	Pos	Pos	Pos
8-154	Human, feces[c]	California	13	Pos	Pos	Pos
D0927-CDC	Human, feces[d]	Mexico	4	Pos	Pos	Pos
D1045-CDC	Human, feces[d]	Texas	4	Pos	Pos	Pos
D3784-CDC	Human, feces[d]	Hawaii	4	Pos	Pos	Pos
D4485-CDC	Human, feces[d]	Minnesota	4	Pos	Pos	Pos
D4911-CDC	Human, feces[d]	Connecticut	4	Pos	Pos	Pos
H4707	Human, feces[e]	California	6A	Pos	ND	ND
H5133	Human, feces[e]	Oregon	6A	Pos	ND	ND
H5134	Human, feces[e]	Oregon	6A	Pos	ND	ND
H6787	Human, feces[e]	Arizona	6A	Pos	ND	ND
D0144-CDC	Human, feces[b]	Switzerland	9A	Pos	ND	ND
D1760-CDC	Human, feces[d]	Texas	9A	Pos	ND	ND
CAHFS-525	Chicken, environment[f]	California	13A	Pos	Pos	Pos
CAHFS-526	Chicken, environment[f]	California	4B	Pos	Pos	Pos
97-1866	Porcine, tissue[g]	Indiana	13A	Pos	Pos	Pos
99-11315	Chicken, egg belt[g]	Nebraska	13A	Pos	Pos	Pos
95-16526	Chicken, egg pool[g]	Washington, D.C.	14B	Pos	Pos	Pos
95-13141	Chicken, egg pool[g]	Maryland	14B	Pos	Pos	Pos
97-637	Porcine, feces[g]	Iowa	8	Pos	Pos	Pos
97-604	Turkey, cloaca[g]	Ohio	8	Pos	Pos	Pos
CAHFS-5	Environment, creek[f]	California	8	Pos	Pos	Pos
CAHFS-184	Chicken, liver[f]	California	4	Pos	Pos	Pos
CAHFS-285	Environment, creek[f]	California	4	Pos	Pos	Pos
CAHFS-320	Chicken, environment[f]	California	4	Pos	Pos	Pos
CAHFS-546	Bovine, milk filter[f]	California	8	Pos	Pos	Pos
426 93-0675	Poultry[b]	France	4	Pos	Pos	Pos
CAHFS-435	Chicken, environment[f]	California	6A	Pos	ND	ND
CAHFS-436	Chicken, environment[f]	California	6A	Pos	ND	ND
CAHFS-739	Chicken, environment[f]	California	6B	Pos	ND	ND

[a] Pos, positive; ND, not determined.
[b] R. Gast, Southeast Poultry Laboratory, USDA, Agricultural Research Service, Athens, Ga.
[c] S. Nunez, Tulare County Public Health, Tulare, California.
[d] T. Barrett, Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, Ga.
[e] B. Holland, Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, Ga.
[f] H. Kinde, California Animal Health and Food Safety Laboratory, San Bernardino Laboratory, San Bernardino, Calif.
[g] K. Ferris, NVSL, Ames, Iowa.

though this primer pair does not detect the strain of serovar Dublin used for the subtraction experiments. Also, this primer pair detected one isolate of *Salmonella* serovar Worthington, while another isolate of the same serovar was not detected. This indicates that there is some degree of diversity within serovars that can be detected by primers from SSH experiments. It is not known if these differences are due to nucleotide differences in the 3′ end of a primer-binding site or whether larger differences are responsible. Another primer pair, to Sdf III, amplified a specific product of the predicted size only with the serovar Enteritidis isolates but amplified other products in six non-Enteritidis isolates (serovars Lomalinda, Mbandaka, Blockley, Derby, Reading, and Kentucky) and produced a smear with one isolate (serovar Berta). Clear positive results were obtained with all 14 serovar Enteritidis environmental, poultry, and other animal isolates tested in this panel (Table 2). The third primer pair that was tested with this panel of strains was that of Sdf I, which yielded remarkably clear results. No products were amplified from the 73 non-Enteritidis isolates, but all 14 serovar Enteritidis isolates showed a clear band of the expected size. Figure 1 shows an Sdf I amplification product for three of the most common phage types of *Salmonella* serovar Enteritidis (lanes 3 to 5), while four other *Salmonella* serovars found in the poultry environment do not show this amplicon (lanes 6 to 9). In addition, two other enteric bacteria, *E. coli* ATCC 25922 and *Citrobacter freundii* ATCC 43864 (lanes 10 and 11) are not detected with this primer pair. The Sdf I primer pair was also tested with other bacteria common in poultry environments, namely, *Proteus mirabilis* ATCC 33946, *Proteus vulgaris* ATCC 13315, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* ATCC 13047, and *Providencia rettgeri* ATCC 29944, and did not show any amplification. The Sdf I, Sdf II, and Sdf III primer pairs were then used to test 37 NVSL phage type reference strains of *Salmonella* serovar Enteritidis (Table 3). The Sdf I sequence was present in all but 6 of 37 phage types (phage types 6A, 9A, 11, 16, 20, and 27). No clinical isolates for phage types 11, 16, 20, and 27 are available from the Centers for Disease Control and Prevention (B. Holland, unpublished data), suggesting that infections from these phage types are exceedingly rare. A subset of these data is presented in Fig. 2A. Amplification of 12 different phage types is shown, with only phage type 6A (lane

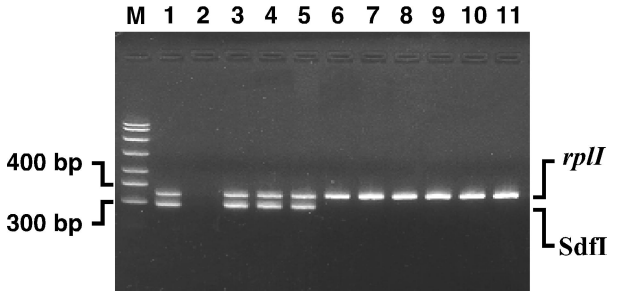

FIG. 1. Specificity of *S. enterica* serovar Enteritidis detection determined using the Sdf I primer pair in PCRs. Lane M, size standards; lane 1, *Salmonella* serovar Enteritidis CAHFS-546 (phage type 8); lane 2, no template; lane 3, *Salmonella* serovar Enteritidis CAHFS-184 (phage type 4); lane 4, *Salmonella* serovar Enteritidis 97-6371A (phage type 8); lane 5, *Salmonella* serovar Enteritidis 97-1866IN (phage type 13A); lane 6, *Salmonella* serovar Pullorum; lane 7, *Salmonella* serovar Typhimurium; lane 8, *Salmonella* serovar Heidelberg; lane 9, *Salmonella* serovar Montevideo; lane 10, *E. coli*; lane 11, *C. freundii*. Amplicons produced by the Sdf I primers (293 bp) and the *rplI* primers (343 bp) are indicated.

7) and 9A (lane 10) showing negative results. Although these results suggest that the Sdf I primers cannot detect other isolates of phage type 6A or 9A, two clinical isolates of phage type 6A (lanes 4 and 5 of Fig. 2B) and phage type 9A (Fig. 2B, lanes 8 and 9) are readily detected with the Sdf I primer pair. Four additional isolates of phage type 6A were also tested and were detected with the Sdf I primers (Table 2). In addition, one isolate of phage type 6B was also detected (Fig. 2B, lane 6). These results suggest that strains that are clearly infectious are detected with the Sdf I primers. Interestingly, the Sdf I and Sdf III primers showed the same pattern when tested with the NVSL strains, except for the NVSL phage type 40 reference strain, raising the possibility that the Sdf I and Sdf III difference fragments may be linked in the *Salmonella* serovar Enteritidis genome. An important difference between the Sdf I and Sdf III primer pairs is that the Sdf III primers generate other products for several of the templates that are not the expected size. The Sdf II primer pair showed amplification with all 37 phage types.

The three primer pairs were also tested against 10 additional serovar Enteritidis clinical isolates taken from stool samples of afflicted humans (Table 2). Eight were phage type 4, one was phage type 7, and one was phage type 13. These 10 samples are geographically diverse, having been collected in Spain, Italy, Mexico, and across the United States from Connecticut to Hawaii. All three primer pairs detected the 10 strains.

Combined with the testing of the phage type 6A, 6B, and 9A strains discussed above, 16 clinical isolates were tested, and all were detected with the Sdf I primers. Thus, these data suggest one highly specific marker for *Salmonella* serovar Enteritidis (Sdf I) has been developed, as well as two other markers that are useful for narrowing *S. enterica* to just a few serovars.

Database searches with the sequences of Sdf I (333 bp), Sdf II (731 bp), and Sdf III (846 bp) showed that positions 5 to 274 of Sdf III, when translated, showed high similarity to the deduced amino acid sequence of a hypothetical protein of the putative cryptic phage CP-933R of *E. coli* O157:H7 strain EDL933 (expected probability of a fortuitous match [E] value 4×10^{-39}). Sdf I and Sdf II showed no similarity to database sequences.

TABLE 3. PCR results for *S. enterica* serovar Enteritidis phage type reference strains

Phage type[b]	Region evaluated[a]		
	Sdf I	Sdf II	Sdf III
1	Pos	Pos	Pos
2	Pos	Pos	Pos
3	Pos	Pos	Pos
4	Pos	Pos	Pos
4A	Pos	Pos	Pos
5	Pos	Pos	Pos
5A	Pos	Pos	Pos
6	Pos	Pos	Pos
6A	Neg	Pos	Neg
7	Pos	Pos	Pos
8	Pos	Pos	Pos
9	Pos	Pos	Pos
9A	Neg	Pos	Neg
9B	Pos	Pos	Pos
10	Pos	Pos	Pos
11	Neg	Pos	Neg
11A	Pos	Pos	Pos
12	Pos	Pos	Pos
13	Pos	Pos	Pos
13A	Pos	Pos	Pos
14	Pos	Pos	Pos
15	Pos	Pos	Pos
16	Neg	Pos	Neg
17	Pos	Pos	Pos
18	Pos	Pos	Pos
19	Pos	Pos	Pos
20	Neg	Pos	Neg
20A	Pos	Pos	Pos
22 SC2	Pos	Pos	Pos
23	Pos	Pos	Pos
24	Pos	Pos	Pos
27	Neg	Pos	Neg
28	Pos	Pos	Pos
31	Pos	Pos	Pos
32	Pos	Pos	Pos
34	Pos	Pos	Pos
40 SC2	Pos	Pos	Neg

[a] Pos, positive; Neg, negative.
[b] NVSL, Ames, Iowa.

Chromosomal localization of the Sdf I locus. To determine whether the Sdf I marker is located on the chromosome or located on a circular plasmid, we developed the following novel assay (Fig. 3). Plasmid-Safe exodeoxyribonuclease from Epicentre Technologies was used to treat plasmid preparations of *Salmonella* serovar Enteritidis CAHFS-285, a phage type 4 isolate. The enzyme digests contaminating chromosomal DNA present in all plasmid preparations but does not affect covalently closed or nicked circular DNAs, i.e., circular plasmids. In addition to the Sdf I primer pair, a primer pair to a known chromosomal gene encoding the L9 ribosomal protein (*rplI*), and a primer pair to a known *Salmonella* plasmid-borne gene, *spvC*, were used as controls. Lanes 1 to 3 show that these primer pairs readily amplify products from total cellular DNA. As expected, all three amplicons were observed in the untreated plasmid preparations (lanes 7 to 9). In exonuclease-treated samples, however, the *spvC* product (lane 6) showed significant amplification, whereas the *rplI* (lane 4) and the Sdf

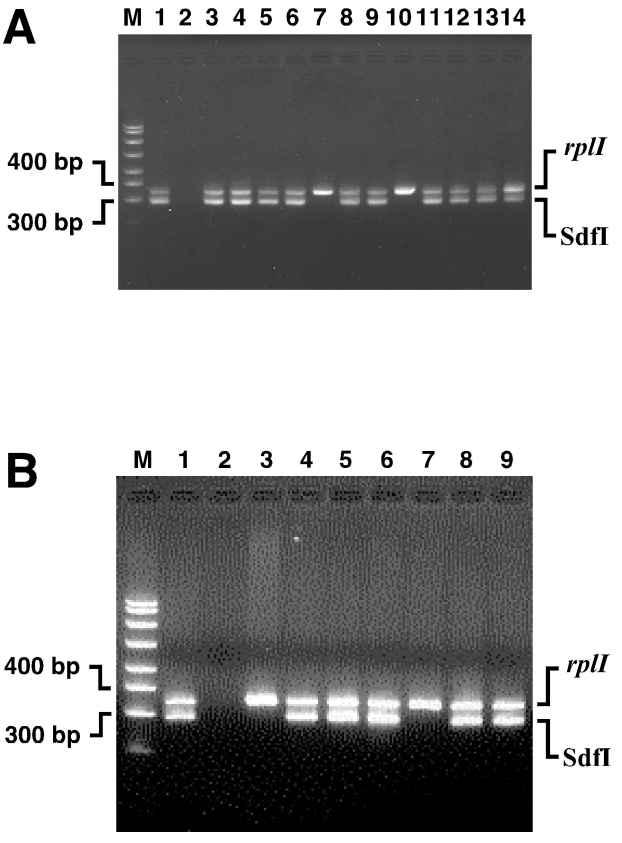

FIG. 2. Specificity of detection of selected *Salmonella* serovar Enteritidis phage type reference strains determined using the Sdf I primer pair in PCRs. The strains used are from the NVSL unless indicated by a specific designation. (A) Detection of Sdf I in phage type reference strains. Lane M, size markers; lane 1, CAHFS-546 (positive control); lane 2, no template; lane 3, phage type 2; lane 4, phage type 3; lane 5, phage type 4; lane 6, phage type 6; lane 7, phage type 6A; lane 8, phage type 8; lane 9, phage type 9; lane 10, phage type 9A; lane 11, phage type 13A; lane 12, 95-13141 (phage type 14B); lane 13, phage type 24; lane 14, phage type 34. (B) Detection of Sdf I in phage type reference strains and clinical strains of phage types 6A, 6B, and 9A. Lane M, size markers; lane 1, CAHFS-546 (positive control); lane 2, no template; lane 3, NVSL 9 (phage type 6A); lane 4, CAHFS-435 (phage type 6A); lane 5, CAHFS-436 (phage type 6A); lane 6, CAHFS-739 (phage type 6B); lane 7, NVSL 13 (phage type 9A); lane 8, D0144-CDC (phage type 9A); lane 9, D01760-CDC (phage type 9A). Amplicons produced by the Sdf I primers (293 bp) and *rplI* primers (343 bp) are indicated.

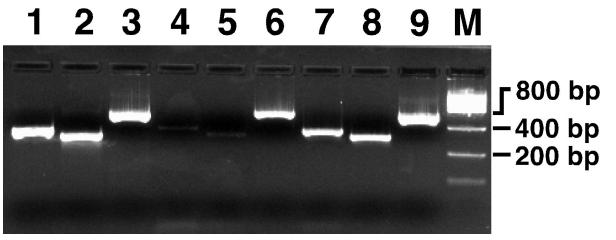

FIG. 3. The *Salmonella* serovar Enteritidis Sdf I region is located on the chromosome. Lane 1, total DNA amplified with *rplI* primers (343-bp amplicon); lane 2, total DNA amplified with the Sdf I primers (293-bp amplicon); lane 3, total DNA amplified with the *spvC* primers (565-bp amplicon); lane 4, plasmid preparation treated with exo-DNase amplified with *rplI* primers; lane 5, plasmid preparation treated with exo-DNase and amplified with Sdf I primers; lane 6, plasmid preparation treated with exo-DNase and amplified with *spvC* primers. Lanes 7, 8, and 9 are the same as lanes 4, 5, and 6, respectively, but without exo-DNase treatment before amplification. Strain CAHFS-285 (phage type 4) was used for these experiments.

I (lane 5) products were only faintly visible. Because the Sdf I signal was reduced similarly to a known chromosomal sequence, this suggests that Sdf I is located on the chromosome.

Cloning of the Sdf I locus. To define the region of the chromosome containing the 333-bp Sdf I SSH product (*Salmonella* difference region I [Sdr I]), a library was constructed using total DNA from the phage type 4 *Salmonella* serovar Enteritidis strain CAHFS-285. A total of 6,528 *E. coli* colonies containing plasmids with 4- to 6-kb inserts, representing >99% of the cellular DNA (assuming a genome size of 5 Mbp), were screened by PCR in pools by using the Sdf I primer pair. One clone was identified, and the complete sequence of its 6,907-bp insert was determined (Fig. 4). There was no similarity to the sequence of Sdf III, which was detected in a similar pattern to that of Sdf I. Sdf I, a *Sau*3AI fragment isolated by SSH, is found between positions 4928 and 5260 of the genomic clone (black region of Fig. 4). Nucleotide sequence comparisons with database sequences showed a near-perfect match at each end to the complete *S. enterica* serovar Typhi genome. On the left end as shown, the match extends from positions 1 to 2101, and on the right end it extends from positions 6160 to 6907. On the left end is a copy of a gene with near-perfect identity to *E. coli ydaO*. Surprisingly, the matches were to two widely separated regions of the serovar Typhi genome (1361375 to 1363475 on the left and 1920934 to 1920189 on the right), suggesting that this region is the site of a major rearrangement with respect to serovar Enteritidis. Overlapping PCR amplifications were used to confirm that the 6,907-bp region of the library clone is contiguous in *Salmonella* serovar Enteritidis and not the result of the ligation of two or more unrelated fragments (data not shown). There are six ORFs of >100 codons in the 4,060-bp novel region (gray and black bars in Fig. 4). We have designated these six ORFs *lygA* to *lygF* for "linked to the *ydaO* gene." These six ORFs encode possible proteins of 207, 105, 173, 155, 119, and 110 amino acids for *lygA* to *F*, respectively. Using a protein BLAST search of the nonredundant database, LygA (positions 2161 to 2784) shows similarity to Exonuclease VIII of *Salmonella* serovar Typhimurium (E value 2×10^{-18}). LygC (positions 3867 to 4388) exhibits weak similarity to phage superinfection exclusion protein B of *E. coli* (E value 6×10^{-5}), while LygD (positions 5036 to 5503) shows even weaker similarity to phage λ repressor cI (E value 10^{-4}). LygF shows some similarity to a hypothetical protein of prophage CP-933R of *E. coli* O157:H7, an enterohemorrhagic strain (E value 10^{-22}). LygE and F overlap to a large extent, which may indicate that one, the other, or both are not genes. The deduced amino acid sequences of *lygB* and *lygE* do not show any similarity to database sequences with a protein BLAST search.

Amplification by PCR was used to examine other areas of the unique region defined by comparison to *Salmonella* serovar Typhi. Primer pairs to *lygA*, *lygC*, and *lygD* were used to amplify sequences from a *Salmonella* serovar Enteritidis phage type 8 strain (CAHFS-546), and a *Salmonella* serovar Dublin strain (CAHFS-9008117D), as well as the library strain, *Salmonella* serovar Enteritidis CAHFS-285 as a positive control. Products

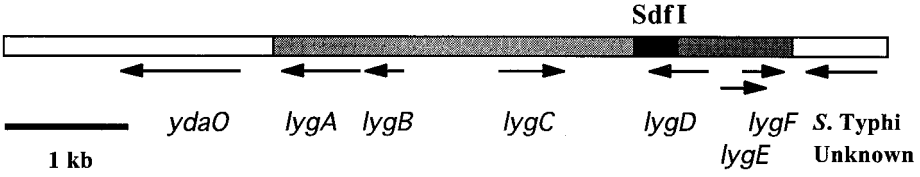

FIG. 4. Chromosomal context of Sdf I. Schematic representation of the Sdf I region from *Salmonella* serovar Enteritidis CAHFS-285 (phage type 4). Open boxes indicate sequence with identity to *S. enterica* serovar Typhi. Gray and black boxes indicate novel sequences. Sdf I, bounded by *Sau*3AI sites, is shown in black. All ORFs of more than 100 codons are indicated with black arrows.

of the expected size were observed in the Enteritidis strains but not in the Dublin strain, a finding consistent with the view that the entire unique region is present in Enteritidis strains but absent in Dublin strains (data not shown). Interestingly, when primers to the nonunique flanking sequences, which would generate an ~4.5-kb amplicon comprising the serovar Enteritidis unique region, were used with the serovar Dublin strain mentioned above, an ~600-bp product was observed. This may indicate that all or most of the unique region is missing in serovar Dublin, and the locus is otherwise colinear. Sequencing the 600-bp amplicon will help to define the precise nature of the difference between the two serovars.

DISCUSSION

Using suppression subtractive hybridization, we found three loci that are restricted to *S. enterica* serovar Enteritidis or are found in a few close relatives. Remarkably, one region, Sdf I, appears to only be found in serovar Enteritidis strains, including a wide range of clinical and environmental samples, and has yielded clear results in laboratory testing. This makes this region an excellent candidate for the detection of serovar Enteritidis within complex samples. Given the wide range of other *Salmonella* serovars and other enteric bacteria found in poultry environments, it is desirable to have markers that will distinguish serovar Enteritidis strains from these bacteria. The Sdf I region appears to satisfy this criterion. It is important to note that in addition to making an excellent marker for nucleic acid detection, this region may also allow the development of an antibody-based test that relies on the detection of one or more putative protein products of the unique ORFs. The extent to which the cloned region varies within serovar Enteritidis strains will be an important question to answer in order to confirm that other areas of this region are useful for detection purposes. A phage type 8 strain was tested with three primer pairs spanning the unique region based on the nucleotide sequence from a phage type 4 strain. The expected products were observed, indicating that these regions were also present in this strain. An ongoing project at the University of Illinois to sequence the genome of the phage type 8 strain LK5 will allow a direct sequence comparison of the Sdf I region from two different strains.

Phage typing is currently the standard method for distinguishing subgroups of serovar Enteritidis (9). This technique has been exploited to ensure that a diverse collection of Enteritidis strains was tested with the diagnostic primer pairs in this study. Using the NVSL reference collection, all 37 phage types were detected with the Sdf I primer pair except phage types 6A, 9A, 11, 16, 20, and 27. Clinical samples for phage types 11, 16, 20, and 27 are not available, indicating that they are not a significant cause of human infections. Although the phage type 6A and 9A reference strains were not detected with the Sdf I primers, two clinical phage type 9A strains and four clinical phage type 6A strains were unambiguously identified by PCR with the Sdf I primer pair. In summary, the Sdf I primer pair clearly detects all strains of a diverse collection of clinical isolates, in addition to detecting all of the environmental isolates tested.

These results demonstrate the lack of a clear relationship between phage typing and the presence of Sdf I. It is possible that subtle differences such as point mutations in the primer binding sites could explain these results. PCR with a primer pair internal to Sdf I, however, showed the same results (data not shown), suggesting that this is not the case. It is also possible that in some reference strains the Sdf I region has been lost during laboratory passage but that selection maintains this region in the natural environment. Cloning and sequencing of the region corresponding to Sdf I from these aberrant strains could help to define the strain differences and perhaps provide insight into this question. Targeted deletion of the region defined by strain comparisons would allow otherwise isogenic strains to be tested to assign a functional role. It is possible that the Enteritidis-specific Sdf I region could be related to one or more of the unique properties of this serovar. Similarity to database sequences is not high enough to provide strong enough evidence to ascribe functions to the putative proteins encoded by this region. The similarity of the *lygF* deduced amino acid sequence to a hypothetical protein of an *E. coli* cryptic phage may suggest, however, that the sequences described here are those of phage remnants. Although Sdf III also showed some similarity to the same cryptic prophage, no Sdf III sequences are present in the 6,907-bp Sdr I in which Sdf I lay, and the degrees of similarity are quite different, so these data do not necessarily imply that these sequences are linked. Another interesting question is whether the phage type 6A and 9A NVSL reference strains, which are Sdf I negative, have the same functional properties, such as chicken colonization, egg infection, and virulence, compared to the phage type 6A and 9A clinical strains, which are Sdf I positive. Importantly, testing of the NVSL reference collection also showed that the most common phage types, i.e., phage types 4, 8, 13, and 13A, were all detected with the Sdf I primer pair. Taken together, the results presented here suggest that Sdf I is a robust marker for pathogenic *Salmonella* serovar Enteritidis strains.

A DNA-based test offers the potential for a significant improvement over current methods of *S. enterica* serovar Enteritidis detection. DNA detection offers the possibility of greater speed, sensitivity, and ease. An important extension of these

studies will be their application to detection in samples taken directly from poultry environments and comparisons to current methods. Combined with improved technology (see, for example, reference 3), it may be possible to perform tests on-site, thus greatly facilitating detection and regular monitoring for serovar Enteritidis.

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