Genetic Determinants and Polymorphisms Specific for Human-Adapted Serovars of *Salmonella enterica* That Cause Enteric Fever

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Salmonella enterica serovars Typhi, Paratyphi A, and Sendai are human-adapted pathogens that cause typhoid (enteric) fever. The acute prevalence in some global regions and the disease severity of typhoidal *Salmonella* have necessitated the development of rapid and specific detection tests. Most of the methodologies currently used to detect serovar Typhi do not identify serovars Paratyphi A or Sendai. To assist in this aim, comparative sequence analyses were performed at the loci of core bacterial genetic determinants and *Salmonella* pathogenicity island 2 genes encoded by clinically significant *S. enterica* serovars. Genetic polymorphisms specific for serovar Typhi (at *trpS*), as well as polymorphisms unique to human-adapted typhoidal serovars (at *sseC* and *sseF*), were observed. Furthermore, entire coding sequences unique to human-adapted typhoidal *Salmonella* strains (i.e., serovar-specific genetic loci rather than polymorphisms) were observed in publicly available comparative genomic DNA microarray data sets. These polymorphisms and loci were developed into real-time PCR, standard PCR, and liquid microsphere suspension array-based molecular protocols and tested for with a panel of clinical and reference subspecies I *S. enterica* strains. A proportion of the nontyphoidal *Salmonella* strains hybridized with the allele-specific oligonucleotide probes for *sseC* and *sseF*; but the *trpS* allele was unique to serovar Typhi (with a singular serovar Paratyphi B strain as an exception), and the coding sequences STY4220 and STY4221 were unique among serovars Typhi, Paratyphi A, and Sendai. These determinants provided phylogenetic data on the genetic relatedness of serovars Typhi, Paratyphi A, and Sendai; and the protocols developed might allow the rapid identification of these *Salmonella* serovars that cause enteric fever.

Enteric or typhoid fever is a systemic disease of humans caused by *Salmonella enterica* serovars Typhi and Paratyphi A. Although enteric fever has largely been eliminated in many parts of the world by improved sanitation, it remains a significant health threat in developing nations (2). Specifically, enteric fever is endemic to Southeast Asia, the Indian subcontinent, and South America and is a growing problem in Africa (10, 18, 20, 41). The worldwide incidence of enteric fever is an estimated 21.6 million cases annually, with 220,000 deaths (6). Notably, the currently used typhoid vaccines and detection methodologies that are based upon the Vi antigen are not appropriate for serovar Paratyphi A; and the reported incidence of this serovar is increasing in some Asian countries, including China, where the numbers of cases caused by serovar Paratyphi A cases exceed those caused by serovar Typhi (2, 30, 43, 46, 47). Industrialized nations are rarely challenged with the treatment of enteric fever, and in those countries cases are usually the result of travelers returning from areas of endemicity (45). In Canada, from 1996 to 2001, serovar Typhi accounted for less than 2% of the total *Salmonella* strains of human origin and serovar Paratyphi A was reported approximately half as frequently (7). However, the emergence of multidrug-resistant strains of serovars Typhi and Paratyphi A is of considerable concern, as the prospect of untreatable enteric fever (at least with affordable therapies) has the potential for

major health impacts in developing nations (33, 44). Novel molecular targets that can be used for the detection of both serovars Typhi and Paratyphi A will be critical for the surveillance and treatment of enteric fever.

Both serovars Typhi and Paratyphi A are highly adapted and can cause only systemic disease in humans. Other subspecies I *S. enterica* serovars can result in nonsystemic disease symptoms in humans and may be commensal or opportunistic pathogens in other warm-blooded animals. Sequencing of the serovar Typhi (strains CT18 and Ty2) and serovar Paratyphi A (ATCC 9150) genomes has revealed significant similarities between the two pathogens (8, 25, 32). Comparative genomic hybridization (CGH) experiments and phylogenetic analyses with *Salmonella* serovars have also demonstrated the genetic relatedness of serovars Typhi and Paratyphi A, even though they are members of different serogroups (serogroups D1 and A, respectively) (4, 34). Genome degradation has led both pathogens to independently accumulate a high proportion of pseudogenes, most of which are required by other *Salmonella* serovars to colonize and invade the gastrointestinal epithelium (25). Gene silencing from pseudogene formation, along with other loss-of-function mutations, has resulted in the adaptation of serovars Typhi and Paratyphi A to a human-specific niche in the last few thousand years (3, 19, 25, 32). The genetic determinants responsible for their systemic nature during human infection have not been readily identified. Serovars Paratyphi B and C are antigenically and genetically distinct from serovars Typhi and Paratyphi A (34, 40), and while all Paratyphi serotypes can result in typhoid-like illness in humans, serovars B and C can also result in zoonotic infections.

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Diagnosis of enteric fever has traditionally been based on blood culture or the Widal test for serum antibodies against O-somatic and H-flagellar antigens, although the latter suffers from substantial variations in interlaboratory specificity and sensitivity (33). Molecular immunology kits that have improved sensitivity and specificity over those of the Widal test are available (31). Once pure cultures have been isolated from clinical samples, serovars Paratyphi A and Typhi are readily identified by Kaufmann-White serotyping (antigenic formulas, 1,2,12:a:[1, 5] and 9,12,[Vi]:d:–, respectively) and by biochemical tests based on the differential production of H₂S and decarboxylation of lysine, with serovar Paratyphi A strains typically being negative for both of these characteristics on lysine-iron agar. PCR-based techniques have been developed to discriminate serovar Typhi from other *Salmonella* serovars; these techniques target the Vi antigen-encoding gene and the flagellin antigen *fliC-d* gene (9, 13, 14, 23, 42). Individually, these targets were not entirely specific for serovar Typhi, including some Vi-negative strains of serovar Typhi that are endemic (1), and neither of these loci are encoded by serovar Paratyphi A. The use of these loci along with O- and H-antigen encoding genes in a five-locus multiplex PCR assay discriminated both serovars Typhi and Paratyphi A from a large panel of *S. enterica* serovars (14). Real-time PCR strategies, however, are a significant improvement over standard PCR methods, leading to more rapid, sensitive, and potentially quantitative results. Recently, real-time PCR methods have been successful in identifying the Vi-antigen gene (9) and estimating serovar Typhi bacterial loads in blood samples (24). Our goal was to identify genetic traits that are characteristic for both serovar Typhi and serovar Paratyphi A by using comparative sequence analyses and to develop real-time PCR and liquid microsphere suspension assays for the molecular identification of these human-adapted typhoidal *Salmonella* strains.

MATERIALS AND METHODS

Bacterial strains. *Salmonella enterica* strains (Table 1) were obtained from the reference stocks of the Enteric Diseases Program at the National Microbiology Laboratory (stock numbers from this source are in the form SXXX or SXXXX, where the X's are numerals); and recent clinical isolates were obtained from the Alberta Provincial Laboratory for Provincial Health, the British Columbia Centre for Disease Control, the Manitoba Cadham Provincial Laboratory, the Ontario Central Public Health Laboratory, the New Brunswick Public Health Branch, or the Laboratoire de Santé Publique du Québec (isolates from these sources have been assigned designations in the form XX-YYYY, where XX represents the last two digits of the year of isolation and YYYY represents a four-digit identifier). Additionally, five *S. enterica* serovar Sendai isolates were provided by the Centers for Disease Control and Prevention (Atlanta, GA), and one serovar Sendai isolate was provided by the Shenzhen Center for Disease Control and Prevention (People's Republic of China). This study focused on *S. enterica* strains that represented the most frequently observed serovars in Canadian clinical laboratories (serovars Enteritidis, Hadar, Typhimurium, Heidelberg, Dublin, Infantis, Newport, Agona, Thompson, Stanley, Reading, Schwarzengrund, Oranienburg, Javiana, and Saint Paul and subspecies I 4,5,12:i:–) and serovars with known genetic relatedness to serovar Typhi or pathogenicity traits similar to those of serovar Typhi (serovars Paratyphi A, Paratyphi B, Paratyphi C, Sendai, Miami, and Muenster).

Publicly available genomic sequence data for the following serovars were used to initiate comparative genomic studies at target loci: *S. enterica* serovar Typhi (strain CT18, GenBank accession number NC_003198; strain Ty2, GenBank accession number NC_004631); *S. enterica* serovar Paratyphi A (strain ATCC 9150, GenBank accession number NC_006511; clinical isolate "Sanger," Sanger Institute Microbial Pathogens Group, unpublished data [http://www.sanger.ac.uk/Projects/Microbes/]); *S. enterica* serovar Paratyphi B (strain SPB7, Genome

TABLE 1. Bacterial strains used in this study

Serovar	No. of strains	Source ^a	Strain identification no.
Typhi	1	AB PLHL	05-0060
	9	NML	S637, S638, S760, S863, S1319, S1403, S1408, S1447, S1522
	2	OPHL	05-0700, 05-1390
Sendai	5	CDC	STK 75, STK 475, 74-1035, 82-0291, 83-0431
	1	NML	S513
	1	Shenzhen	CS1
Paratyphi A	1	AB PLHL	04-7611
	1	MB CPL	03-6479
	3	NML	S350, S440, S1089
	1	NB PHB	03-7699
Paratyphi B	1	BCCDC	05-1920
	6	NML	S363, S428, S867, S938, S1583, S1610
Paratyphi C	3	NML	S877, S878, S1058
Typhimurium	11	NML	S442, S450, S834, S862, S1304, S1543, S1549, S1553, S1577, S1605, S1623
Enteritidis	2	NML	S413, S1275
	1	NB PHB	05-2165
Dublin	1	NML	S1189
Heidelberg	1	MB CPL	05-1049
	4	OPHL	04-3194, 04-5511, 04-1511, 03-4690
	5	QPHL	04-3293, 04-4717, 04-5435, 04-0346, 03-4601
Hadar	1	AB PLHL	05-2137
	1	NML	S1628
Infantis	1	AB PLHL	05-2138
	1	NML	S1627
Newport	1	NML	S1550
Thompson	1	NML	S786
Agona	5	NML	S1017, S1084, S1545, S1571, S1538
Javiana	1	NML	S539
St. Paul	1	NML	S1598
Miami	2	NML	S1016, S1128
Muenster	3	NML	S1049, S1130, S1248
Oranienburg	1	NML	S407
Stanley	1	NML	S372
Schwarzengrund	1	NML	S1582
Reading	1	NML	S405
Subspecies I 4,5,12:i:–	1	NML	04-3408

^a AB PLPH, Alberta Provincial Laboratory for Public Health; BCCDC, British Columbia Centre for Disease Control; CDC, Centers for Disease Control and Prevention; MB CPL, Manitoba Cadham Provincial Laboratory; NML, National Microbiology Laboratory standard strain; NB PHB, New Brunswick Public Health Branch; OPHL, Ontario Public Health Laboratory; QPHL, Laboratoire de Santé Publique du Québec; Shenzhen, Shenzhen Centre for Disease Control and Prevention (China).

TABLE 2. Oligonucleotides used in this study

Target ^a	Oligonucleotide	Sequence (5' to 3') ^b	Platform
<i>sseC</i>	GIL286	GCGAAGAAGTGAGCGAAAGT	PCR/sequencing
<i>sseC</i>	GIL287	TCCCTCAGTAAGGCATACAGG	PCR/sequencing
<i>sseC</i>	GIL287-L	Biotin-ZOOOTCAGTAAGGCATACAGG	PCR/Luminex
<i>sseC</i> (typhoidal)	<i>sseC</i> _1073RL	cgcaaACAGAACTTTTCCCCTTCTGc[FAM]G	LUX RT-PCR ^d
<i>sseC</i> (typhoidal)	<i>sseC</i> _953FU	GGGCGAGATGGAAGAGGAGTC	LUX RT-PCR
<i>sseC</i> (typhoidal)	DOB75	ATATGACAAAAAATGCGGGAA	Luminex probe
<i>sseF</i>	GIL284	TGAAAATTTCATATTCCGTCAGC	PCR/sequencing
<i>sseF</i>	GIL285	AGCAATGGGTAACTCTGCTC	PCR/sequencing
<i>sseF</i> (typhoidal)	GIL278	AGCGGCAAGCAATATAGTCG	PCR
<i>sseF</i> (typhoidal)	GIL279	GTAAGCACACAGGAAGTGT	PCR
STY4220 (typhoidal)	GIL282	AGGCGGTGATACTGATACGG	PCR/sequencing
STY4220 (typhoidal)	GIL283	TATTGACGAAATGCCACAGC	PCR/sequencing
STY4221 (typhoidal)	4221_559FL	cggcAGGAAGTCAGGAAGTCGc[FAM]G	LUX RT-PCR
STY4221 (typhoidal)	4221_577RU	TTCGTGAGAAATCAGGATTGTTCC	LUX RT-PCR
STY4221 (typhoidal)	GIL280	AGGTCTGTTGCTGGACTGAAG	PCR/sequencing
STY4221 (typhoidal)	GIL281	TTCAGGACAACTGCCATCTG	PCR/sequencing
<i>trpS</i>	GIL259	TGTACCAAAACCAATCTGGTGC	PCR/sequencing
<i>trpS</i>	GIL260	ATATCCAGCAGGTTAGAGAC	PCR/sequencing
<i>trpS</i>	GIL260-L	Biotin-FZFZCCAGCAGGTTAGAGAC	PCR/Luminex
<i>trpS</i>	DOB52	CGTTTTAACGGGCTGTATGG	5'-Nuclease RT-PCR
<i>trpS</i>	DOB53	GACCGCAGCTTTGATTTCT	5'-Nuclease RT-PCR
<i>trpS</i> (serovar Typhi)	ST1	[TET]AACCGTAATAACGTAATCGGCCTGTT ^c	5'-Nuclease RT-PCR
<i>trpS</i> (serovar Typhi)	DOB78	CGTAATCGGCCTGTTGG	Luminex probe

^a The targeted serovar or group of serovars (typhoidal indicates serovars Typhi, Paratyphi A, and Sendai) is indicated in parentheses.

^b FAM, 6-carboxyfluorescein; TET, tetrachlorofluorescein; O, C-phosphorothioate; F, A-phosphorothioate; Z, T-phosphorothioate; lowercase bases at the 5' end indicate those required for LUX primer hairpin formation and are not present in the target sequence; the penultimate 3' base in lowercase is labeled with a fluorophore, indicated in brackets.

^c A 6-carboxytetramethylrhodamine quencher is present at the 3' terminus.

^d RT-PCR, real-time PCR.

Sequencing Center at Washington University Medical School, unpublished data [http://genomeold.wustl.edu/projects/bacterial/sparatyphiB/index.php]); *S. enterica* serovar Typhimurium (strain LT2, GenBank accession number NC_003197; strains DT104 and SL1344, Sanger Institute, unpublished data); *S. enterica* serovar Enteritidis (strain PT4, Sanger Institute, unpublished data; strain LK5 [strain PT8], University of Illinois at Urbana—Champaign, unpublished data [www.salmoneella.org]); and *S. enterica* serovar Choleraesuis (strain SC-B67, GenBank accession number NC_006905).

PCR and sequencing. Template DNA was prepared by centrifuging 1 ml of log-phase cultures grown in brain heart infusion broth, resuspending the pellet in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (Sigma, St. Louis, MO), and boiling for 10 min. The boiled cell debris was pelleted, and the supernatant was removed and used as the DNA template in real-time and standard PCRs.

Standard PCR was performed with Platinum High Fidelity *Taq* (Invitrogen, Burlington, Ontario, Canada) following the manufacturer's directions. The oligonucleotides are described in Table 2. PCR conditions were as follows: initial denaturation at 94°C for 5 min and 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 30 s, with a final extension at 68°C for 5 min. The PCR products were purified by using a QIAquick PCR purification kit (QIAGEN, Mississauga, Ontario, Canada) and were sequenced by using the same primers used to generate this template. Sequencing was performed on an ABI 3730 instrument (Applied Biosystems, Foster City, CA).

Light Upon eXtension (LUX) fluorogenic and unlabeled primer pairs were designed by using D-LUX designer software (Invitrogen) by targeting polymorphic regions in target loci that were characteristic for different *S. enterica* serovars. For LUX real-time PCR, Platinum quantitative PCR Supermix UDG (Invitrogen) was used for the amplification mixture, with each LUX primer used at a final concentration of 200 nM and with 2 µl of template used, for a total reaction volume of 25 µl. Real-time PCRs were performed on a SmartCycler 2.0 instrument (Cepheid, Sunnyvale, CA). Samples were amplified by an initial denaturation at 95°C for 3 min and 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 15 s, and an extension step at 72°C for 15 s. Fluorescence was detected at the annealing step, and the threshold level was set at 30 fluorescence units. A real-time PCR result was considered positive at the point that the fluorescent signal exceeded the background level (the cycle threshold value).

For 5'-nuclease real-time PCR, primers and probes (Table 2) were designed with Applied Biosystems Primer Express software, version 2.0. TaqMan Universal PCR Master Mix and No AmpErase UNG (Applied Biosystems) were used

as the amplification mixture, with the final concentrations of the 5'-nuclease probes and primers (Operon Biotechnologies Inc., Huntsville, AL) being 125 nM and 800 nM, respectively. The total reaction volume was 25 µl, including 2.5 µl of template. The DNA was amplified in a SmartCycler 2.0 instrument by an initial denaturation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s and a single annealing-extension step at 60°C for 60 s. Fluorescence was detected during the annealing-extension step, and the threshold level was set at 30 fluorescence units.

Liquid microsphere suspension arrays. Allelic discrimination of *trpS* and *sseC* was achieved after PCR amplification of biotin-labeled target DNA from representative strains with a GeneAmp 9700 thermocycler (Applied Biosystems), primers GIL259 and GIL260-L (*trpS*) or primers GIL286 and GIL287-L (*sseC*) (Table 2), and the thermocycling parameters detailed above, except that 35 cycles were performed. The PCR mixtures were purified with QIAquick DNA purification kits (QIAGEN) and eluted with 50 µl of EB buffer (QIAGEN). Oligonucleotides GIL260-L and GIL287-L contain four bases with phosphorothioate linkages, as well as a biotin molecule, all at the 5' end. Between the two strands of the target DNA, the strands produced from GIL259 and GIL286 (*trpS* and *sseC* "sense" strands) are sensitive to T7 exonuclease digestion, whereas the antisense strands are protected due to the phosphorothioate linkages (12, 29). DNA digestion was performed by mixing 43 µl of purified PCR product with 5 µl of buffer 4 and 2 µl of T7 exonuclease (both from New England Biolabs, Ipswich, MA) (20 U total) and incubating at 37°C for 1 h. T7 exonuclease was inactivated by adding 2 µl of 0.5 M EDTA (Ambion, Austin, TX). Selective degradation ensures elimination of the unlabeled target DNA strand, thereby preventing reannealing between the two target DNA strands during hybridization, which, if left intact, would limit the intended hybridization to that between the biotin-labeled strand and the *trpS* or *sseC* allele-specific probes coupled to microspheres in subsequent steps.

Oligonucleotide probes were designed by matching the sense strand in regions characteristic for individual allele subtypes. Oligonucleotides were screened for potential secondary structures or cross-hybridization between probes by using SBEprimer software (16). The oligonucleotide probes were synthesized with a 5' C-12 amine and coupled to xMAP-carboxylated fluorescently coded microspheres (Luminex Corporation, Austin, TX). Microsphere sets 103 and 108 were coupled to oligonucleotides DOB75 and DOB78, respectively (Table 2); and hybridization of biotin-labeled *trpS* and *sseC* target DNA strands to the capture probe-coupled microspheres and flow cytometry were performed in triplicate, as described previously (12). The positive cutoff value for the *sseC* assay was chosen

FIG. 1. Comparative genomic analyses of the *S. enterica* core locus *trpS*, which encodes tryptophanyl-tRNA synthetase. (A) Phylogeny of clinically significant *S. enterica* serovars based upon a neighbor-joining tree; the scale of the distance score is represented by a horizontal bar. (B) Split decomposition of *trpS*; recombination between loci is indicated when the topology resembles a network, rather than the single branching points seen in normal tree topologies; the scale of the distance score is represented by a horizontal bar. (C) Multiple-sequence alignment of the *trpS* segment containing potential serovar-specific polymorphisms; strain identification is indicated in parentheses, and where sequence data from multiple strains from a single serovar were identical, no strain identifier was included; the 5'-nuclease probe is identified with a horizontal line under the corresponding sequence, and the microsphere-coupled probe is identified with a horizontal line labeled with a circle. The GenBank accession numbers for multiple sequenced loci are presented in the Materials and Methods.

to be a value 10 times greater than the value for the negative control; for the *trpS* assay the positive cutoff value was chosen to be a value 2.5 times greater than the value for the negative control, accounting for the lower overall signal strength of the true-positive signals.

Bioinformatics. Initial screening of polymorphic loci was performed with BLASTn, and position-specific iterated basic local alignments of the STY4217 to STY4222 gene products were performed with PSI-BLAST (www.ncbi.nlm.nih.gov/BLAST/). Pairwise global DNA sequence alignments were performed with Align (http://www.ebi.ac.uk/emboss/align/), multiple-sequence alignments were completed with ClustalW (www.ebi.ac.uk/clustalw/) and Boxshade (www.ch.embnet.org), neighbor-joining trees were constructed with the Hasegawa-Kishino-Yano (HKY85) distance correction with SplitsTree4 (15), and genetic diversity statistics were calculated with DnaSP 4.10.3 (37). Split decomposition analysis was performed with SplitsTree4 by using the alignment inputs created by ClustalW, and the calculations used only parsimony-informative sites. Both neighbor-joining and split decomposition trees were calculated by using only those segments of each locus for which data for all strains examined were available (i.e., regions from the complete genome data outside of the amplified segments of each locus were not included). Artemis (38) was used for calculation of the G+C content and visualization of the annotated features of the *Salmonella* chromosomal segments.

Nucleotide sequence accession numbers. The sequence data from this study were deposited in GenBank under accession numbers DQ320510 to DQ320558 and DQ451529 to DQ451530.

RESULTS

Identification of genetic traits unique to human-adapted typhoidal *Salmonella*. Serotype-specific genetic polymorphisms unique to serovar Typhi or unique to human-adapted typhoidal *Salmonella* serovars Typhi and Paratyphi A were putatively identified by using the genomic sequence data for *S. enterica* serovars Typhi (strains CT18 and Ty2), Paratyphi A (strains ATCC 9150 and a clinical isolate being evaluated at the Sanger Institute), Paratyphi B (strain SPB7), Typhimurium (strains LT2, DT104, and SL1344), Enteritidis (phage type 4 and strain LK5), and Choleraesuis (strain SCB67) (5, 8, 25, 26, 32). Our initial target loci included core bacterial determinants (such as *trpS*, *dnaX*, and *ftsZ*) that are effective for predicting the genetic relatedness between species but that are not subject to frequent mutation (48). Discrete regions within these loci that might encode sufficient genetic diversity for molecular identification of serovars (to be observed as conserved serotype-specific nucleotide polymorphisms) were investigated by performing multiple-sequence alignments for all accessible alleles

TABLE 3. Genetic diversity of *trpS*, *sseC*, and *sseF*

Target ^a	No. of sequences	No. of serovars ^b	Size of target (bp)	No. of polymorphic sites (π) ^c	No. of synonymous polymorphic sites	No. of nonsynonymous polymorphic sites	dN/dS ratio
<i>trpS</i> (full CDS)	6	6	1,005	32 (0.012)	31	1	0.008
<i>trpS</i> (amplicon)	15	13	167	10 (0.014)	10	0	0.000
<i>sseC</i> (full CDS)	6	6	1,455	77 (0.027)	35	42	0.362
<i>sseC</i> (amplicon)	16	14	269	38 (0.067)	13	25	0.611
<i>sseF</i> (full CDS)	6	6	783	37 (0.023)	18	19	0.370
<i>sseF</i> (amplicon)	15	13	241	22 (0.035)	16	6	0.161

^a Full CDSs are from the genomic sequences available at GenBank (see Materials and Methods); amplicons were produced with primers designed for conserved regions in GenBank-available sequences and were sequenced by using these same primers.

^b Minimally includes serovars Typhi, Paratyphi A, Paratyphi B, Typhimurium, Choleraesuis, and Enteritidis.

^c π , a measure of genetic diversity.

of these target loci (data not shown). The sequences of *trpS*, which encodes tryptophanyl-tRNA synthetase, identified two closely spaced polymorphic sites that discriminated serovar Typhi from the other serovars examined (Fig. 1).

The prospect of molecular serotyping for Shiga toxin-producing *Escherichia coli* has been initiated by using a polymorphic genetic determinant encoding the type III secretion system effector protein EspZ (12). Polymorphisms in *espZ* that correlated to serotype lineages may have arisen due to the functional adaptation of this virulence determinant to host structures (12, 17); therefore, determinants encoding *Salmonella*-specific type III secreted effector proteins were investigated for polymorphisms that correspond to the human-adapted typhoidal *Salmonella*. This included coding sequences from the *Salmonella* pathogenicity island 2, and comparative analyses of the sequenced strains identified that both *sseC* and *sseF* (encoding type III secretion system effector proteins) are variable between serovars (Table 3). Both *sseC* and *sseF* encoded distinct clusters of polymorphic sites that were putatively unique to *S. enterica* serovars Typhi and Paratyphi A but absent in the sequenced strains of serovars Typhimurium, Enteritidis, Choleraesuis, and Paratyphi B (Fig. 2 and 3).

Large data sets from genomic sequencing and CGH projects have provided a wealth of information on the genetic contents of related bacterial strains. The overall genetic relatedness and contents of 79 *S. enterica* subspecies I strains (representing 27 serotypes) were analyzed by CGH (34), and in this data set a region comprising the serovar Typhi strain CT18 coding sequences STY4219 to STY4222 was identified as "present" only in serovars Typhi and Paratyphi A. These coding sequences appear to be encoded in a single operon comprising coding sequences STY4217 to STY4222 that was inserted into an ancestral *S. enterica* sequence, as indicated by the lower G+C content of this segment compared to those of the adjacent chromosomally encoded determinants of serovar Typhi strain CT18 (32).

PCR-based detection of serovars Typhi and Paratyphi A. To confirm the presence of the STY4217 to STY4222 region in additional serovar Typhi and Paratyphi A strains and to examine additional serovars, we developed LUX real-time PCR primers for STY4221 (Table 2; Fig. 4). The LUX system requires only one fluorogenic primer, which is self-quenching through the formation of a hairpin loop, and one unlabeled primer (28). As a more economical alternative, standard PCR primer pairs were also developed for the detection of STY4220 (186-bp product) and STY4221 (264-bp product), and our

panel of strains were examined for the presence of these genes (Table 4). Both STY4220 and STY4221 were detected only in serovars Typhi, Paratyphi A, and Sendai; and all strains of these particular serovars were positive. Notably, *S. enterica* serovar Sendai is also a human-adapted serovar that can result in systemic, typhoid-like disease symptoms (40), although it is rarely observed in clinical laboratories. Within the previously published CGH data, these genes were observed to be absent in serovar Sendai reference strain SARB58 (34); however, the accuracy of a PCR assay directed against a single locus is possibly higher than that of an individual probe in a CGH experiment comprising a whole genome. A total of seven serovar Sendai strains (originating from Canada, the United States, and China) were screened, and all strains were positive by the STY4220 and STY4221 PCR assays. Sequence analysis of a STY4221 amplicon overlapping the LUX primer-binding sites identified a single conserved polymorphism in the serovar Sendai and Paratyphi A strains compared to the sequences of the serovar Typhi strains, but this position was outside of the primer-binding sites (data not shown).

The serovar-specific sites encoded within *trpS*, *sseC*, and *sseF* had the potential to be used for allelic discrimination; and 5'-nuclease real-time PCR (*trpS*), LUX real-time PCR (*sseC*), and standard, nonfluorogenic PCR (*sseF*) assays were attempted. The 5'-nuclease system uses two unlabeled primers (which serve as forward and reverse PCR primers) and an allele-specific oligonucleotide probe coupled with both a fluorophore and a quencher (21). The 5'-nuclease *trpS* probe was designed against the serovar Typhi *trpS* allele, with the two characteristic sites represented in the central region of the oligonucleotide (Fig. 1; Table 2). The LUX primer set designed for *sseC* included four characteristic sites conserved among typhoidal *Salmonella* strains in the forward primer (Fig. 2). The standard PCR primers designed for *sseF* included five conserved sites putatively characteristic for typhoidal *Salmonella* strains in the reverse primer (Fig. 3; Table 2).

To determine the success of these primer and probe designs, real-time or standard PCRs were performed with template DNA from serovars Typhi and Paratyphi A. An expanded panel of serovars with known pathogenic or genetic similarities to serovars Typhi and Paratyphi A, as well as strains representing the most commonly observed *S. enterica* serovars in Canadian clinical laboratories, was also examined (Table 4; Fig. 4). The *trpS* probe was successful in differentiating serovar Typhi from all other serovars examined except for a single Paratyphi B isolate, S1583. The LUX-*sseC* reaction was posi-

FIG. 2. Comparative genomic analyses of the *S. enterica* pathogenicity island 2 locus *sseC*. (A) Phylogeny and (B) split decomposition of *sseC*; see the Fig. 1 legend for further details. (C) Multiple-sequence alignment of the *sseC* segment containing potential serovar-specific polymorphisms; strain identifications are indicated in parentheses, and where sequence data from multiple strains from a single serovar were identical, no strain identifier was included; LUX real-time PCR primers are identified by a horizontal lines with half arrowheads under the corresponding sequence, and the microsphere-coupled probe is identified with a horizontal line labeled with a circle. The GenBank accession numbers for previously sequenced loci are presented in the Materials and Methods.

tive with all serovar Typhi, Paratyphi A, and Sendai strains. A LUX-*sseC* product was also generated with all serovar Agona, Oranienburg, Reading, Javiana, and Paratyphi C strains examined and a single serovar Paratyphi B isolate, S1583 (Fig. 4;

Table 4); however, in all instances the product formed more than seven cycles after the serovar Typhi product formed. The *sseF* PCR was positive with serovars Typhi, Paratyphi A, Sendai, Infantis, and Paratyphi B isolate S1583 (Table 4).

FIG. 3. Comparative genomic analyses of the *S. enterica* pathogenicity island 2 locus *sseF*. (A) Phylogeny and (B) split decomposition of *sseF*; see the Fig. 1 legend for further details. (C) Multiple-sequence alignment of the *sseF* segment containing potential serovar-specific polymorphisms; strain identifications are indicated in parentheses, and where sequence data from multiple strains from a single serovar were identical, no strain identifier was included; PCR primers are identified by horizontal lines with half arrowheads under the corresponding sequence. The GenBank accession numbers for previously sequenced loci are presented in the Materials and Methods.

Liquid microsphere suspension arrays for *trpS* and *sseC*. To investigate supplemental or improved allelic discrimination methods, microsphere-oligonucleotide probe conjugates targeting serovar Typhi-specific alleles (*trpS*) and human-adapted typhoidal serovars (serovars Typhi, Paratyphi A, and Sendai; *sseC* alleles) were developed (Fig. 1, 2, and 5). The Luminex microsphere suspension array technology uses allele-specific oligonucleotide probes conjugated to fluorescently coded microspheres to capture soluble DNA in a liquid phase and characterize hybridization partners with flow cytometry. Target DNA for the *trpS* and *sseC* loci was generated by using biotinylated PCR primers with internal phosphorothioate linkages

(Table 2) (see Materials and Methods), and the region of each locus amplified for liquid microsphere suspension assays corresponded to the same region subjected to sequence analysis.

The *trpS* microsphere-bound probe was designed against a region similar to that against which the 5'-nuclease probe was designed, and this hybridized to the target DNA produced from serovar Typhi isolates, as well as the single Paratyphi B isolate, S1583 (Fig. 5; Table 4). The *sseC* probe was designed against a region different from that against which the *sseC* LUX primers were designed (which weakly detected serovars Agona, Javiana, Oranienburg, Reading, and Paratyphi C) and included five centrally located sites putatively unique to the

TABLE 4. Summary of allelic discrimination for *trpS*, *sseC*, and *sseF* by PCR, real-time PCR, and liquid suspension microsphere arrays

Serovar	No. of strains	PCR ^b						Microsphere arrays ^b		
		5'-Nuclease for <i>trpS</i> (Typhi)	LUX ^c		Standard		<i>sseF</i> (typhoidal)	No. of strains	<i>trpS</i> (Typhi)	<i>sseC</i> (typhoidal)
			<i>sseC</i> (typhoidal)	STY4221	STY4221	STY4220				
Typhi	12	+	+	+	+	+	+	3	+	+
Sendai	7	—	+	+	+	+	+	1	—	+
Paratyphi A	6	—	+	+	+	+	+	1	—	+
Paratyphi B	6	—	—	—	—	—	—	1	—	+
Paratyphi B ^a	1	+	+/-	—	—	—	+	1	+	+
Paratyphi C	3	—	+/-	—	—	—	—	1	—	—
Enteritidis	3	—	—	—	—	—	—	1	—	—
Heidelberg	10	—	—	—	—	—	—	1	—	—
Newport	1	—	—	—	—	—	—	1	—	—
Typhimurium	11	—	—	—	—	—	—	2	—	—
Agona	5	—	+/-	—	—	—	—	1	—	—
Muenster	3	—	—	—	—	—	—	1	—	—
Miami	2	—	—	—	—	—	—	1	—	—
Hadar	2	—	—	—	—	—	—	1	—	—
Dublin	1	—	—	—	—	—	—	1	—	—
Thompson	1	—	—	—	—	—	—	1	—	—
Javiana	1	—	+/-	—	—	—	—	1	—	—
St. Paul	1	—	—	—	—	—	—	1	—	—
Infantis	2	—	—	—	—	—	+	1	—	—
Stanley	1	—	—	—	—	—	—	1	—	—
Oranienburg	1	—	—	—	—	—	—	1	—	—
Schwarzengrund	1	—	+/-	—	—	—	—	1	—	—
Reading	1	—	—	—	—	—	—	1	—	—
Subspecies I 4,5,12:i:—	1	—	—	—	—	—	—	1	—	—

^a *S. enterica* serovar Paratyphi B isolate S1583.

^b Typhi, designed to detect serovar Typhi-specific polymorphisms; typhoidal, designed to detect polymorphisms specific for human-adapted serovars (serovars Typhi, Paratyphi A, and Sendai); +, the cycle threshold value was reached for LUX or 5'-nuclease real-time PCR, a product was detected in standard PCR, or the mean fluorescence during microsphere suspension array was distinguishably higher than that for the negative control.

^c +/-, positive cycle threshold values were obtained, but at cycles later than those for strains with + values (Fig. 4).

typhoidal serovars (Fig. 3). Accordingly, this probe was able to distinguish serovars Typhi, Sendai, and Paratyphi A from serovar Agona and other serovars (Fig. 5). This site was also conserved among all serovar Paratyphi B strains examined, including isolate S1583, which each had a mean fluorescence significantly above the background levels in the *sseC* microsphere suspension assay, although not to the same extent as those of the other typhoidal serovars (Fig. 5). No additional target regions that could discriminate serovar Typhi from serovar Paratyphi B and the other serovars examined were observed in the *sseC* sequence data.

Comparative analyses of the *trpS*, *sseC*, *sseF*, and STY4217 to STY4222 sequences. To confirm the genotypes of all serovars producing positive reactions (serovars Typhi, Paratyphi A, Sendai, Agona, Javiana, Paratyphi C, Infantis, Oranienburg, Reading, and Paratyphi B strain S1583), the corresponding regions of *trpS*, *sseC*, and *sseF* were sequenced (Fig. 1 to 3). We also sequenced these loci of additional strains of serovars

Typhimurium, Muenster, Heidelberg, and Dublin to provide insight into the relationship between the genotype and the PCR results, as well the phylogenetic associations among *S. enterica* serovars. Primers to amplify and sequence each locus were designed for conserved sites identified within multiple-sequence alignments of the available *S. enterica* sequence data (Table 2). The selected region of each allele comprised the putative serovar-specific polymorphisms and other variable sites; and the amplicon lengths for *trpS*, *sseC*, and *sseF* were 334 bp, 295 bp, and 278 bp, respectively. These data indicated that serovars producing positive PCR or microsphere array reactions encoded sites identical or similar to the targeted alleles of *trpS*, *sseC*, and *sseF* (Fig. 1 to 3).

The genetic diversity and number of synonymous and non-synonymous mutations were calculated for the *trpS*, *sseC*, and *sseF* loci by using the sequence data from the amplicons produced in this study and the publicly available complete coding sequence (CDS) data from reference strains (Table 3). At the

FIG. 4. Allelic discrimination of serovar Typhi and typhoidal serovar-specific polymorphisms and detection of loci unique to human-adapted typhoidal serovars by real-time PCR. (A) 5'-Nuclease real-time PCR characterization of *trpS* alleles; (B) LUX real-time PCR detection of STY4221; (C) LUX real-time PCR characterization of *sseC* alleles. The strains examined are indicated in the legend insets. NTC, no-template control. Positive experimental reactions and the no-template control reaction in each data set have solid lines connecting the datum points, whereas the negative experimental reactions are indicated with dashed lines. In the *sseC* LUX assay, positive experimental reactions where the product was detected more than seven cycles later than serovar Typhi was detected are indicated with shaded markers at each datum point. The primers are described in Table 2.

FIG. 5. Allelic discrimination of serovar Typhi and human-adapted typhoidal serovar-specific polymorphisms by liquid microsphere suspension arrays. Biotin-labeled template (*trpS* [A] and *sseC* [B]) was amplified from strains of the indicated serotypes and incubated with fluorescently coded microspheres coupled with an oligonucleotide probe targeting the serovar-specific alleles. The background fluorescence contributed by the microsphere-probe mixture was determined by using a no-template control (TE buffer), and the standard errors are indicated by vertical lines on each bar.

trpS, *sseC*, and *sseF* loci, the sequenced amplicon represented 17%, 19%, and 31% of the complete CDSs, respectively; but these segments accounted for 31%, 49%, and 60% of the total polymorphic sites encoded in the complete CDSs, respectively (Table 3). Notably, the cellular function of each gene product was reflected in the ratio of the number of nonsynonymous mutations to the number of synonymous mutations (dN/dS). At the *trpS* locus, which encodes a core bacterial determinant essential for the translation module, a single nonsynonymous site was observed, whereas the *sseC* and *sseF* loci, both of which encode secreted virulence proteins, had more nonsynonymous sites than synonymous sites (Table 3). The higher dN/dS likely reflects the adaptive nature of these gene products to host structures during pathogenesis. Notably, none of the targeted alleles of *trpS* (serovar Typhi) or *sseC* and *sseF* (serovars Typhi, Paratyphi A, and Sendai) have been subjected to recombination, as suggested by split decomposition analysis (Fig. 1 to 3).

The island from STY4217 to STY4222 was inserted between *yhiI* and *yhiN* genes (present as adjacent CDSs in *S. enterica* serovar Typhimurium strain LT2, STM3587 to STM3588), and this genetic layout is similar to that observed in serovar Typhi strain Ty2 (CDSs t3930 to t3934) and serovar Paratyphi A strain ATCC 9150 (CDSs SPA3439 to SPA3443). Bioinformatic analyses of this region did not identify any obvious virulence factors or indicate the possible origin. Each of the individual coding sequences from STY4217 to STY4222 had other possible orthologues encoded by enteric proteobacteria, as detected by PSI-BLAST analysis (data not shown); but no currently sequenced genome encoded contiguous coding sequences that had a gene order similar to that of this operon or a protein sequence identity with this operon (other than the serovar Typhi strain Ty2 and the serovar Paratyphi A genomes). Because of the conservation and exclusivity of the island from STY4217 to STY4222 to the human-adapted typhoidal *Salmonella* strains, the island provides tremendous promise for use in the identification of systemic serovars by molecular tests that target this region.

DISCUSSION

Enteric fever represents a considerable health threat to travelers and residents of regions of endemicity. The rapid identification of etiological agents in clinical samples and pure culture is important for disease surveillance and the response to endemic, outbreak, and sporadic occurrences. To achieve this goal, we developed PCR and microsphere suspension array protocols to differentiate serovars Typhi and Paratyphi A from other *Salmonella enterica* serovars. Comparative sequence analyses presumptively identified two conserved polymorphisms in *trpS* unique to serovar Typhi and discrete regions within *sseC* and *sseF* characteristic of serovars Typhi and Paratyphi. Additional sequencing at the *sseC* and *sseF* loci revealed that these same distinguishing sites are encoded in serovar Sendai strains. Serovar Sendai is also a human-adapted typhoidal *S. enterica* serovar, although it is infrequently recorded in clinical laboratories. The allele-specific real-time PCR probe for *trpS* successfully differentiated serovar Typhi, whereas primers targeting the serovar Typhi-Paratyphi A-Sendai alleles of *sseC* and *sseF* also generated products with nontyphoidal serovars; but *sseC* was successfully used in a liquid microsphere-oligonucleotide suspension assay to identify the typhoidal serovars. A unique genetic island encoded by serovars Typhi and Paratyphi A was identified from a previously published microarray data set (34); and by the use of PCR assays directed against STY4220 and STY4221, we detected this island in all other Typhi, Paratyphi A, and Sendai strains examined but in no other *S. enterica* serovars. These assays, in particular, those targeting *trpS* and those targeting STY4220 and STY4221, provide the means for the molecular detection of serovar Typhi and the detection of all human-adapted typhoidal serovars, respectively.

Serovar Typhi is one of the most genetically distinct but homogeneous serovars of *S. enterica*, as observed by multilocus enzyme electrophoresis (36, 40), multilocus sequence typing (19), protein profiling (11), and plasmid analysis (22). Comparative

genomic analyses with DNA microarrays have also identified that serovars Typhi, Paratyphi A, and Sendai have similar genetic contents but are diverse from the other *S. enterica* serovars (4, 34). Serovars Sendai and Paratyphi A also share many serological characteristics, with both serovars having similar O antigens and identical phase 1 and 2 flagellar antigens (40). The genetic relatedness of serovars Typhi, Paratyphi A, and Sendai was represented in the characteristic polymorphisms of the *sseC* and *sseF* alleles, as well as the presence of the entire island from STY4217 to STY4222. The *sseC* and *sseF* loci are among the most polymorphic in pathogenicity island 2; but the alleles of each locus encoded by serovars Typhi, Paratyphi A, and Sendai were 99.6 to 100% identical. These alleles were each distinct from those encoded by other serovars, paralleling the overall genetic relatedness of *S. enterica* serovars; and the prevalence of nonsynonymous mutations in *sseC* and *sseF* might be reflective of host adaptation. The cellular function contributed by the island from STY4217 to STY4222 was not obvious after comparison to other annotated bacterial genomes; but coinherence of this region suggests that serovars Typhi, Paratyphi A, and Sendai have a common ancestor. The two polymorphic sites observed in *trpS* and considered unique to serovar Typhi are unlikely to contribute to the pathogenesis of this serovar, but these sites signified divergence between serovars Typhi and Paratyphi A outside of the pseudogenes. Notably, recombination between serovars was indicated at each locus examined by split decomposition analysis but was not indicated at the serovar Typhi-encoded alleles of *trpS* nor at the serovar Typhi-, Paratyphi A-, and Sendai-encoded alleles of *sseC* and *sseF*. A lack of recombination might indicate functional constraint on further mutation of these alleles. Comparative genomic analyses of a group of serovars with related pathogenicity traits (such as the human-adapted typhoidal *Salmonella*) therefore identified lineage-specific polymorphisms and coding sequences, and these determinants might be responsible for the pathogenicity traits (or are minimally coinherited with the determinants that are responsible).

The PCR-based allelic discrimination assays for *trpS*, *sseC*, and *sseF* detected all intended serovars (serovar Typhi and/or Paratyphi A), but at each locus other strains or serovars had positive reactions. This included a single serovar Paratyphi B strain (S1583) with positive reactions for all three loci; serovars Paratyphi C, Agona, Oranienburg, Reading, and Javiana with positive reactions for *sseC*; and serovar Infantis with a positive reaction for *sseF* due to identical or nearly identical sequences at the real-time primer and probe binding sites (Fig. 1 to 3). Notably, the subpopulation of serovars that produced *sseC* at later cycle thresholds than serovar Typhi (more than seven cycles) each encoded polymorphisms at the 5' ends of the primer-binding sites (in relation to the serovar Typhi sequences). This indicates that LUX primers can tolerate mismatches outside the 3' regions but that product is not formed at the same rate as template without mismatches. The specificity at *sseC* was improved by targeting an alternate site in the microsphere suspension assay; and by that assay only serovars Typhi, Paratyphi A, Paratyphi B, and Sendai were detected. Serovar Paratyphi B isolate S1583 was *d*-tartarate positive (indicative of biovar Java). This serovar normally causes nonsystemic disease symptoms (35), so it was surprising that there was considerable similarity to the serovar Typhi-encoded al-

les of *trpS*, *sseC*, and *sseF*. Notably, there are extensive genetic differences within strains of serovar Paratyphi B (27, 35, 39), so the observation of a single strain with alleles similar to those of the human-adapted serovars might be possible. Otherwise, use of the island from STY4217 to STY4222 for molecular identification of human-adapted typhoidal serovars resolved the cross-reaction with strain S1583, as this region was not encoded in any serovar Paratyphi B isolate. Additionally, since this region was encoded exclusively in all strains of serovars Typhi, Paratyphi A, and Sendai examined, the STY4220 and STY4221 CDSs were ideal markers for the detection of human-adapted typhoidal *Salmonella*, whereas serovars Paratyphi A and Sendai would not be detected by use of the current *fliC-d*- or Vi antigen-specific reagents.

The endemic nature of serovar Typhi throughout a significant proportion of the world, the emergence of antibiotic resistance, and the rising incidence of serovar Paratyphi A in Asia demand the development of sensitive molecular protocols and vaccines suitable for the identification and prevention of typhoidal *Salmonella* infections other than just those caused by serovar Typhi (2, 30, 47). Although real-time PCR offers a rapid method for the detection of serovars Typhi and Paratyphi A and this platform can readily be deployed in outbreak situations, the expense and availability of the equipment required remain limiting factors in its widespread use in routine surveillance worldwide. Accordingly, standard PCR assays with nonfluorogenic oligonucleotides were also developed in this study. These molecular methods were tested only with pure cultures of *S. enterica*; and it will be necessary to test these reagents with human clinical samples such as blood, urine, and stool to determine if typhoidal *Salmonella* can be detected without the initial requirement of microbial culturing or examination of circulating antibodies. If these protocols can successfully identify serovars Typhi, Paratyphi A, and Sendai (especially in blood, which is one of the first sites from which these organisms can be isolated from during illness), then incidences of enteric fever caused by *S. enterica* could be more accurately diagnosed and the worldwide surveillance of these pathogens would be greatly enhanced.

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