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Precise Excision of the Large Pathogenicity Island, SPI7, in *Salmonella enterica* Serovar Typhi

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The large pathogenicity island (SPI7) of *Salmonella enterica* serovar Typhi is a 133,477-bp segment of DNA flanked by two 52-bp direct repeats overlapping the *pheU* (phenylalanyl-tRNA) gene, contains 151 potential open reading frames, and includes the *viaB* operon involved in the synthesis of Vi antigen. Some clinical isolates of *S. enterica* serovar Typhi are missing the entire SPI7, due to its precise excision; these strains have lost the ability to produce Vi antigen, are resistant to phage Vi-II, and invade a human epithelial cell line more rapidly. Excision of SPI7 occurs spontaneously in a clinical isolate of *S. enterica* serovar Typhi when it is grown in the laboratory, leaves an intact copy of the *pheU* gene at its novel join point, and results in the same three phenotypic consequences. SPI7 is an unstable genetic element, probably an intermediate in the pathway of lateral transfer of such pathogenicity islands among enteric gram-negative bacteria.

Epidemic recurrences of typhoid fever, caused by *Salmonella enterica* serovar Typhi, remain among the most costly human infections in terms of both morbidity and mortality (44). *S. enterica* serovar Typhi is transmitted by contaminated water and food and is an exclusively human pathogen. As with many bacterial infections, the treatment of *S. enterica* serovar Typhi infection has proven difficult due to the recent emergence of multidrug-resistant strains (54).

S. enterica serovar Typhi is closely related to *S. enterica* serovar Typhimurium, which is among the model eubacteria that can be manipulated rapidly and easily by powerful genetic methods, including phage-mediated genetic exchange or generalized transduction, which was discovered in *S. enterica* serovar Typhimurium (68). *S. enterica* serovar Typhimurium can be isolated from a variety of mammals, birds, and reptiles. It has a wide host range and causes a lethal systemic infection in mice yet usually results only in a limited gastroenteritis in humans. In contrast, *S. enterica* serovar Typhi causes a lethal systemic infection in its exclusively human source and host.

To determine the genetic basis of this difference in host range, we are testing the hypothesis that the larger differences between the genomes of these two serovars contribute to their different host ranges. Comparison of the genome sequences of *S. enterica* serovars Typhi and Typhimurium shows that more than 80% of their sequences are more than 95% identical. They differ primarily by blocks of genes unique to each serovar (7, 12, 17, 18, 45, 48). The largest difference between these genomes is a 133.5-kb region that includes the genes required for the biosynthesis of the capsular antigen, Vi (36). This region is called *Salmonella* pathogenicity island 7 (SPI7), or the large pathogenicity island (PI), because has many features in common with PIs found in other gram-negative enteric pathogens (22). SPI7 has a G+C base composition (49%) significantly different from that of the entire *S. enterica* serovar Typhi genome (52%), it is bounded by direct repeats overlapping a tRNA gene (*pheU*), and it contains genes encoding known pathogenicity determinants, including SopE (24) and type IV pili (64, 65).

In this work, we show that *S. enterica* serovar Typhi SPI7, like many other PIs, is an unstable genetic element that undergoes excision, resulting in profound genotypic and phenotypic changes. These phenotypic changes include the loss of Vi antigen production, which, in turn, results in an altered colony morphology and resistance to phage Vi-II. The latter change in phenotype has allowed us to show that excision of SPI7 occurs spontaneously in a clinical isolate of *S. enterica* serovar Typhi when it is grown in the laboratory and to develop a combined genetic selection and screen to measure the frequency of SPI7 excision. The companion paper (42) presents independent and complementary evidence that SPI7 is an unstable genetic element prone to excision.

(A portion of the results presented here, showing that clinical isolates of *S. enterica* serovar Typhi are variable in genome structure, was presented at the 45th Annual Meeting of the Sociedad de Biología de Chile in November 2002 and has been published in abstract form.)

MATERIALS AND METHODS

Bacteria and bacteriophage strains, media, and growth conditions. *S. enterica* serovar Typhi clinical strains are from the Infection Disease Hospital Lucio Cordova in Santiago, Chile, and have been described previously (55). Each strain was isolated directly from blood samples of different patients being treated for typhoid fever. *S. enterica* serovar Typhi Ty2 is from the Instituto de Salud Publica, Santiago, Chile, and has been used to prepare the *S. enterica* serovar Typhi vaccine against Vi antigen available in Chile. Serovars of *S. enterica* subspecies I are from the *Salmonella* reference collection B (SARB) (6). Strains of *S. enterica* were growth routinely in Luria-Bertani (LB) medium (Bacto Tryptone, 10 g/liter; Bacto Yeast Extract, 5 g/liter; NaCl, 5 g/liter) at 37°C, with aeration provided by shaking, or anaerobically prior to invasion assays with cultured human cells (14). When required, medium was supplemented with

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TABLE 1. Oligonucleotide primers used in this study

Primer	Nucleotide sequence	Coordinates[a]
A	GCTCCCACATAACGGCCAGCATCAA	4408179–4408203
B	TGCGGTGAAACCTGCTGTGCCA	4409757–4409736
C	GAGCGGTAATGCCCGAGTCCTGCA	4540805–4540828
D	TTTGCACAGTGCCGTCAGGGTGGAG	4543298–4543274
E	CGAATTGTCAAGACCCTGAGCGTGA	4530038–4530062
I43aD	AGGCGTAGTGAATGCAGCAAAGCGC	4414160–4414184
I43aR	AGGCCACACGAACCCCTCGGGTATT	4416883–4416859
I43bD	CCCCCTCTCTGTTATGCCGTCTGCA	4424290–4424313
I43bR	ACCGCCACCCAGCAGGAAATTACCC	4427171–4427147
I43bD2	TTTCTGCGTTGTGGGGTTCTGGGC	4433591–4433614
I43bR2	CCACCCGATCAAGCAAGGCAAGATG	4436745–4436721
I43bD3	ATAAGCCCCTCACCGGAAACCCGTC	4454572–4454596
I43bR3	CAGCACACGGGATTTCAGGCCTGTC	4457403–4457379
sopED2	GCTGACTTTGGTGCTGCTGCTCTCG	4479697–4479721
sopER2	CTGGCGTATGCGGGGTCTTTACTCG	4482122–4482098
I45D1	TCTCGCACTTTTTCGGGATGCTGA	4512443–4512466
I45R1	GCAACCATGCGAACCCATTCATTC	4515144–4515121
I45D2	TTCAGATACGCTACCGCCCCTGGC	4523950–4523973
I45R2	GGGGCTGGATCAGAAGCTCGTGCAA	4526942–4526918
R1(H1+P1)	TGGTTATGGGGAGGTGAGCTATGGCTAAAGCACCAC TGTTAA*TGTAGGCTGGAGCTGCTTCG*[b]	4412019–4412060
R1(H2+P2)	AAACAACCCGTTAGTATTCTAGGCTGGCGAAGGTTTTCGTCT*CATATGAATATCCTCCTTAG*[b]	4420739–4420698

[a] Coordinates are those of the *S. enterica* serovar Typhi CT18 sequence (45).
[b] Italics indicate the region that anneals to the 5′ or 3′ end of a kanamycin cassette flanked by the FRT sites in plasmid KD4.

kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), tetracycline (20 μg/ml), ampicillin (100 μg/ml), glucose (Glc; 0.2%, vt/vol), and/or arabinose (Ara; 0.4%, wt/vol). Media were solidified by the addition of agar (15 g/liter).

Vi-II phage was provided by Stanley Maloy. High-titer stocks of a spontaneous clear-plaque-forming mutant of this phage were grown by inoculating liquid cultures of *S. enterica* serovar Typhi STH2370 R1::*kan* grown to an optical density at 600 nm of 0.25 in 50 ml of LB medium supplemented with 100 mM $MgCl_2$ and 5 mM $CaCl_2$ with 10^{10} PFU of the phage. Mixtures were incubated for 6 h with aeration, lysed by the addition of 5 ml of $CHCl_3$, and centrifuged for 20 min at 7,000 × *g* to remove cellular debris. Supernatants were sterilized by the addition of 1 ml of $CHCl_3$, and titers in the stocks were determined using the soft-agar overlay method.

PCR amplifications and DNA sequence analyses. PCR amplifications were performed using an Eppendorf thermal cycler and *Taq* DNA polymerase (Fermentas) or ThermalAce (Invitrogen) in a standard volume of 25 μl. Reaction mixes contained 1 × buffer, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at 200 μM, 1 μM each primer, 100 ng of template DNA, and 2 U of DNA polymerase. Standard conditions for amplification were 30 cycles of incubation at 96°C for 40 s, 60°C for 40 s, and 72°C for 3 min, followed by a final extension step at 72°C for 10 min. Template *S. enterica* serovar Typhi chromosomal DNA was prepared as described previously (55), and the sequences of the primers used are listed in Table 1. Sequencing of the amplified PCR products from SARB50, STH2327, Ty2, and STH2370ΔSPI7 genomic DNA templates across SPI7 deletion join points was performed by the Center of DNA and Peptide Sequencing at Pontificia Universidad Católica de Chile.

Construction and analysis of *S. enterica* serovar Typhi mutant STH2370 R1::*kan*. A mutant derivative of *S. enterica* serovar Typhi strain STH2370 with a substitution of Kan^r for exclusive region R1 of SPI7 was made by the method of Datsenko and Wanner (16). PCR primers (60 bp) R1(H1+P1) and R1(H2+P2) were designed with 40 bp of 5′ homology to the *S. enterica* serovar Typhi CT18 sequence and 20 bp of 3′ homology complementary to plasmid pKD4 and were used to amplify this plasmid as the template. Plasmid pKD46 was introduced into STH2370 by electroporation, and recombinants were recovered in LB medium with ampicillin and Glc at 30°C. Cells were grown at 30°C in LB medium with ampicillin and Ara, made electrocompetent, and electroporated with approximately 500 ng of the PCR product; the cells were plated on LB medium with kanamycin at 37°C to select for the substitution and cure plasmid pKD46. The presence of the substitution mutations in recombinant strains resulting from this procedure was confirmed by PCR amplification, using primers complementary to the *S. enterica* serovar Typhi genome, flanking the site of substitution.

Agglutination assays. Agglutination tests were performed on glass microscope slides by mixing 100 μl of anti-Vi serum (Becton Dickinson and Co.) with single colonies resuspended in 100 μl of 0.85% NaCl. Reactions were visualized by phase-contrast microscopy.

Invasion assays. *S. enterica* serovar Typhi strains were grown in LB medium anaerobically at 37°C to an optical density at 600 nm of 0.2. Monolayers of HEp-2 cells were grown in 96-well plates to 80% confluence in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a 10% CO_2–90% air atmosphere. Approximately 5×10^6 *S. enterica* serovar Typhi cells were used to infect monolayers of HEp-2 cells at a multiplicity of infection of 100:1, and entry of bacteria into epithelial cells was quantified by the modified gentamicin protection assay (14). The percent invasion was calculated as 100 times the ratios of the titers of gentamicin-resistant bacteria to the total bacteria added.

RESULTS

SPI7 is found only in serovars Typhi and Paratyphi C. Comparison of the genome sequences of type strains of the related pathogens *S. enterica* serovar Typhi (45) and *S. enterica* serovar Typhimurium (37) shows that these genomes have a common genomic blueprint but differ by deletions, insertions, and substitutions of blocks of genes that may be among the determinants of host specificity (18). The largest of these differences, SPI7, is a contiguous region of 134,477 bp that represents the genes numbered STY4521 through STY4680 in the reference genome sequence of *S. enterica* Typhi CT18 (23, 45, 64, 65). In place of *S. enterica* serovar Typhi SPI7, the *S. enterica* serovar Typhimurium genome has a 1.5-kb region that includes a single gene predicted to encode a repressor in the TetR family (Fig. 1).

As we show below, SPI7 is an unstable genetic element. It is flanked by 52-bp direct repeats. The right direct repeat (*attR*) overlaps the 3′ end of the *pheU* gene (STY4681), and the left direct repeat (*attL*) overlaps an incomplete copy of this gene, adjacent to the *phoN* gene (STY4519). The STY4680 gene, internal to SPI7 and immediately upstream of *attR*, is predicted to encode an integrase in a large family of proteins related to temperate coliphage P4 integrase. Genes encoding members of this family of integrases are associated with many other pathogenicity islands found in the genomes of related gram-negative pathogens (9).

We analyzed the distribution of SPI7 among different serovars of *S. enterica* in two ways. First, we took advantage of the fact that the draft or complete genome sequences of five other

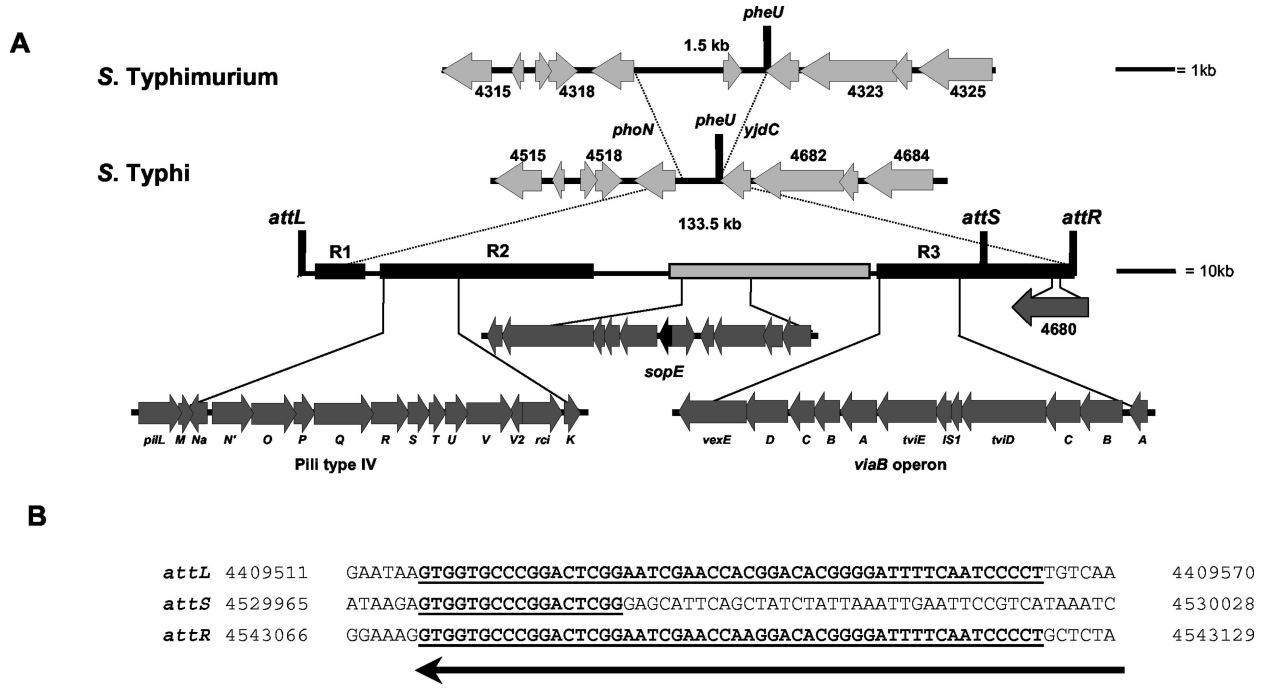

FIG. 1. Structure of *S. enterica* serovar Typhi SPI7. (A) Alignment of the segment of the *S. enterica* serovar Typhi CT18 genome sequence (45) containing SPI7 with the corresponding segment of the *S. enterica* serovar Typhimurium LT2 genome (37). Gene numbers (STM and STY, respectively) are as designated in the annotations of these genome sequences. In place of the 133.5-kb SPI7 in the *S. enterica* serovar Typhi genome, *S. enterica* serovar Typhimurium has a 1.5-kb segment of DNA. SPI7 is flanked on the left by the *phoN* gene and the incomplete 3′ end of the *pheU* gene and on the right by the complete *pheU* and adjacent *yjdC* genes; other gene numbers shown are from the annotated genome sequences. SPI7 has three regions of genes, R1, R2, and R3 (solid boxes), that are not found in other serovars of *S. enterica*, with the exception of serovar Paratyphi C (see the text). Region 2 includes the genes involved in the synthesis of type IV pili (*pilL-pilK*), and region 3 includes the Vi antigen biosynthetic (*viaB*) operon (*vexE-tviA*). A copy of the *sopE* gene is found between regions 2 and 3, flanked by genes similar to a subset of phage P2 genes. As described in the text, this region also includes genes predicted to encode homologues of plasmid transfer factors and of phage P4 proteins. Among the genes similar to those of phage P4 is STY4680, located adjacent to the right end (*attR*) of SPI7 and predicted to encode an integrase. (B) Comparison of the 52-bp left and right repeat sequences (*attL* and *attR*) flanking SPI7 with the short 17-bp repeat sequence (*attS*) found downstream of the *viaB* operon. The arrow at the bottom indicates the 3′ end of the *pheU* gene; coordinates (given as base pairs in the text) are those of the *S. enterica* serovar Typhi CT18 sequence (45).

S. enterica serovars (Paratyphi A, Paratyphi B, Enteritidis, Dublin, and Pullorum) have been determined. We asked whether SPI7 is present in these genomes and found that it has three contiguous regions of sequence (designated R1, R2, and R3 in Fig. 1A) that are unique to serovar Typhi and are not present in the genome sequences of these other *S. enterica* serovars, nor in that of serovar Typhimurium.

Region 1 is 7.4 kb long (bp 4412234 to 4419352 in the *S. enterica* serovar Typhi CT18 sequence) and includes seven open reading frames with, as yet, no assigned functions. Region 2 is 35.7 kb long (bp 4423666 to 4459383) and includes at least 42 open reading frames, 15 of which are involved in the assembly of type IV pili (64). The predicted products of three open reading frames downstream of these genes (STY4554, STY4562, and STY4573) have weak similarities to the products of the R64 *traE*, *S. enterica* serovar Typhi R27 *traG*, and *Helicobacter pylori virB4* genes (23.7% identity in 219 amino acids, 25.9% identity in 630 amino acids, and 18.6% identity in 354 amino acids; GenBank accession no. BAA77990, AAF69957, and AAD06494; respectively), which may encode proteins involved in conjugal DNA transfer (29, 31, 33, 63). Region 3 is 32.8 kb long (bp 4510289 to 4543125) and includes at least 30 open reading frames, 10 of which comprise the *viaB* operon, responsible for the production of the Vi capsular antigen (26).

Region 3 also contains a 17-bp repeat sequence, *attS* (GTG GTGCCCGGACTCGG; bp 4489517 to 4409533), found in the same orientation within both of the 52-bp direct repeats flanking SPI7. Gene STY4666, immediately upstream of *attS*, is predicted to encode an integrase similar to the *Pseudomonas aeruginosa* XerC protein and shares conserved active-site residues critical for integrase function (19, 43, 47). Between regions 2 and 3 is a 33.5-kb stretch of DNA (bp 4473830 to 4507770) which includes the *sopE* virulence gene; this region is also found in the draft *S. enterica* serovar Paratyphi A genome sequence and includes genes similar in sequence to those of temperate phages P2.

To extend this comparative genomic analysis, we tested for the presence of SPI7 in 32 different representatives of serovars of *S. enterica*. We used PCR to amplify subregions of the three regions of SPI7 that initially appeared to be unique to *S. enterica* serovar Typhi by our whole-genome sequence comparisons. Six primer pairs (see Materials and Methods) were designed to amplify internal segments of each of these three regions, and a seventh primer pair was designed to detect the

presence of the *sopE* gene (Fig. 2). We found that none of the seven primer pairs yielded PCR products with template DNAs from 31 of these serovars. Only DNA prepared from an isolate of serovar Paratyphi C (strain SARB48) yielded amplification products with each the first six primer pairs. These results are consistent with those of a previous report showing that *S. enterica* serovar Paratyphi C has the *viaB* operon and makes Vi antigen (25). We did not obtain an amplification product with *S. enterica* serovar Paratyphi C DNA and the seventh primer pair designed to detect the *sopE* gene, suggesting that a portion of SPI7 including *sopE* is not present in the genome of this *S. enterica* serovar Paratyphi C isolate.

SPI7 has a variable structure in clinical isolates and laboratory strains of *S. enterica* serovar Typhi. The genome sequences of independent isolates of gram-negative pathogens, including *S. enterica* serovar Typhi, differ significantly from one another (7). Presumably, the genetic variability among different isolates of the same gram-negative pathogen is due to selective pressures imposed by a changing environment, which enrich for a diversity of genome structures. Unlike isolates of many other serovars of *S. enterica*, isolates of serovar Typhi differ from one another by multiple chromosomal inversions, most of which appear to have arisen due to homologous recombination events between its seven rRNA operons (35, 36). In addition, independent isolates of *S. enterica* serovar Typhi differ from one another by smaller genome rearrangements, as well as insertions and deletions (7, 36). Many of these intraspecific insertions and deletions, like the interspecific differences between isolates of *S. enterica* serovars, may reflect the presence or absence of prophages, whose integration and excision are dependent on site-specific recombination events (18, 35, 36, 48).

Like many integrated prophages, SPI7 is bounded by direct repeats of a portion of a tRNA gene (11), one of which is adjacent to a putative integrase gene, STY4680. Because many prophages are in a dynamic equilibrium between integrated and excised states, we used two different methods to test whether SPI7 can undergo site-specific recombination between these direct repeats (*attL* and *attR*). We tested whether SPI7 is present in a variety of independent isolates of *S. enterica* serovar Typhi, and we tested whether SPI7 could be excised from a clinical isolate of *S. enterica* serovar Typhi by site-specific recombination between its *attL* and *attR* sites.

To determine if SPI7 is ubiquitous among independent isolates of *S. enterica* serovar Typhi, we used the PCR test described above to detect its presence in 27 clinical isolates and in the laboratory strain Ty2. All seven primer pairs yielded PCR products of the predicted sizes when genomic DNA isolated from 25 of the 27 clinical isolates was used as the template (Fig. 2). In contrast 2 of the 27 isolates (STH2327 and STH2361) consistently failed to yield products after amplification with each of these primer pairs. In addition, we did not obtain products when we used DNA isolated from laboratory strain Ty2 as the template.

We confirmed the presence or absence of SPI7 in these strains by Southern hybridization. Three of the PCR products described above were labeled and used to probe Southern blots of genomic DNA from the isolates of *S. enterica* serovar Typhi. Each probe hybridized with specific restriction fragments of genomic DNA prepared from each of the strains, with the exception of DNAs prepared from strains STH2327, STH2361, and Ty2 (data not shown).

Because SPI7 is present in some, but not all, clinical isolates of *S. enterica* serovar Typhi, we also tested whether it is present in two additional independent isolates of *S. enterica* serovar Paratyphi C, SARB49 and SARB50, by repeating our PCR test. One strain (SARB49) yielded products with five of the seven primer pairs but not with primer pairs designed to amplify regions of SPI7 containing the *pilV*, *pilV2*, and *rci* genes (bp 4433592 to 4436746) or the *sopE* gene. The other strain (SARB50) failed to yield products with each of the seven primer pairs (data not shown). These results show that the presence and structure of SPI7 are variable among independent isolates of both serovars Typhi and Paratyphi C.

Because SPI7 is bounded by direct repeats adjacent to a putative integrase gene, one simple hypothesis to account for the majority of these results is that SPI7 can undergo precise excision due to site-specific recombination between these direct repeats. To test this hypothesis, we designed a second PCR experiment. In this experiment, we used pairs of four different primers, A, B, C, and D, to amplify template DNAs isolated from representative isolates of *S. enterica* serovar Typhi with and without SPI7 (Fig. 3). These primers correspond to regions within the *phoN* gene upstream of the left SPI7 boundary (primer A), within SPI7 downstream of its left boundary (primer B), within SPI7 upstream of its right boundary (primer C), and within adjacent DNA downstream of the right SPI7 boundary (primer D). If a strain has SPI7, amplification of *S. enterica* serovar Typhi chromosomal DNA with primer pairs AB and CD should yield products of 1.6 and 3.0 kb, including *attL* and *attR*, respectively. If a strain has undergone recombination to generate a precise deletion of SPI7, primer pair AD should yield a 2.0-kb product including the deletion join point, *attB*.

As shown in Fig. 3, amplification of chromosomal DNA from strain STH2370 by primer pairs AB and CD yielded products of sizes corresponding to the SPI7/chromosome junctions including *attL* and *attR* whereas amplification of the STH2327 and STH2361 templates yielded no products. In contrast, amplification of STH2327 and STH2361 DNAs by primer pair AD yielded a 2-kb product. The sequence of the product from strain STH2327 shows that it includes the novel join point, *attB*, predicted to result from *attL* × *attR* site-specific recombination (GenBank accession no. AY391281).

Surprisingly, strain Ty2 yielded a product only with primer pair CD, indicating that it retained the left, but not the right, SPI7/chromosome junction. This result suggests that this strain has lost only a portion of SPI7. As described above, SPI7 contains a short 17-bp repeat, *attS*, near the right end of SPI7, also found within the 52-bp *attL* and *attR* sequences flanking SPI7. Therefore, we considered the possibility that Ty2 has a deletion of the majority of SPI7 resulting from site-specific recombination between *attL* and *attS*. To test this possibility, we amplified DNA isolated from Ty2 with a primer (primer E) complementary to sequences immediately downstream of *attS* and primer A, immediately upstream of *attL*. This amplification yielded a 1.4-kb product (Fig. 3), whose sequence includes the novel join point arising from recombination between *attL* and *attS* (GenBank accession no. AY391279). Presumably, in strain Ty2, *attL* × *attS* site-specific recombination has resulted

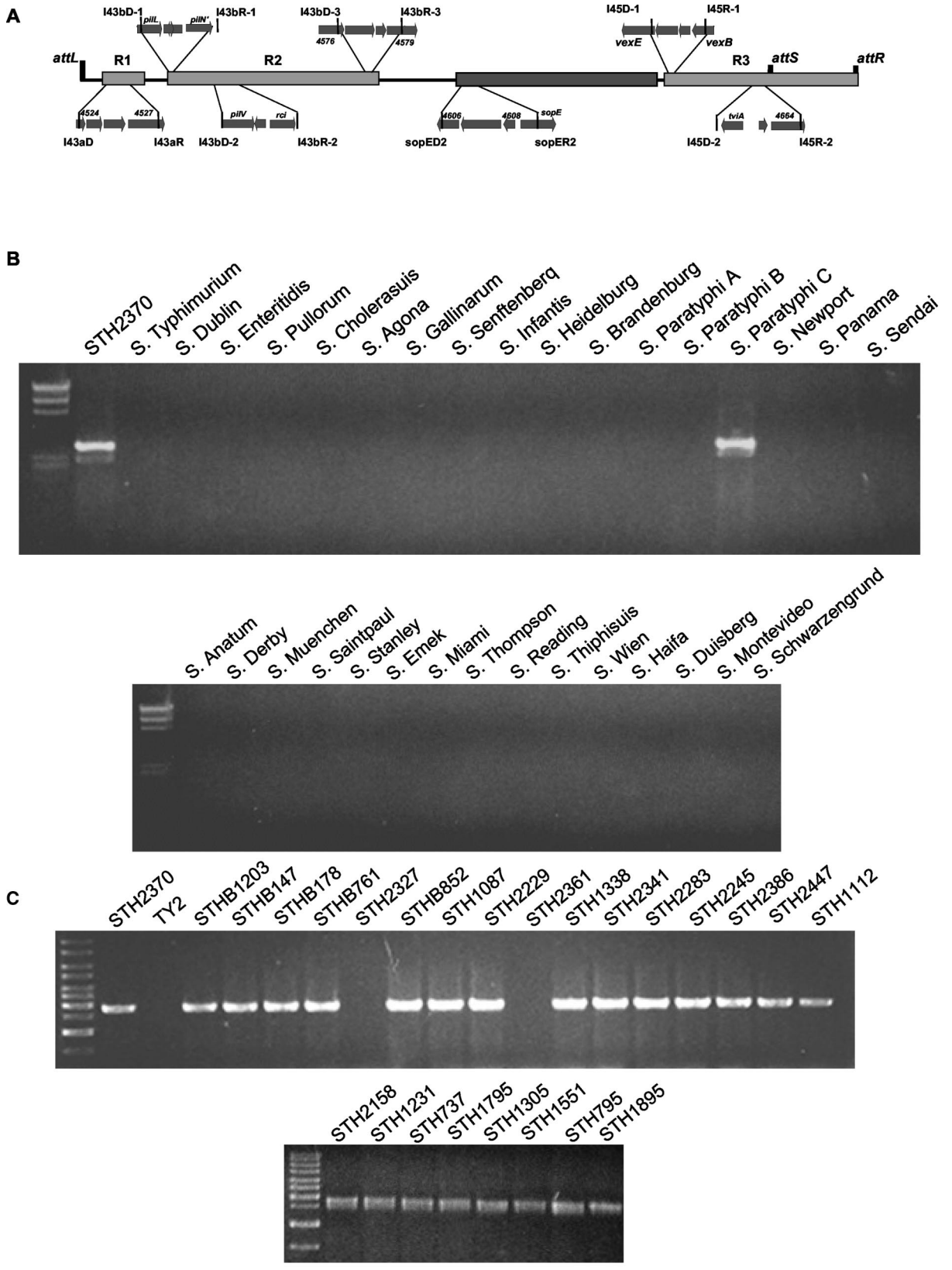

FIG. 2. SPI7 is found in only two serovars of *S. enterica*, Typhi and Paratyphi C, and is not present in a subset of *S. enterica* serovar Typhi clinical isolates. (A) Positions of the primers used to amplify subregions of SPI7. Each primer pair (D [direct] and R [reverse]) was designed to amplify a <3-kb subregion of SPI7 present in the *S. enterica* serovar Typhi CT18 sequence (45); positive results for the majority of these amplifications show that SPI7 is present in most clinical isolates of *S. enterica* serovar Typhi used in this study. (B) PCR was used to amplify a region internal to region 2 of SPI7 (bp 4454572 to 4457403) in isolates of different serovars of *S. enterica*. Only isolates of serovars Typhi and Paratyphi C yielded products after amplification with this primer pair corresponding to the *S. enterica* serovar Typhi CT18 sequence. (C) SPI7 is found in a majority of clinical isolates of *S. enterica* serovar Typhi. Similar results were obtained with five additional primer pairs designed to amplify subregions of regions 1, 2, and 3 (data not shown).

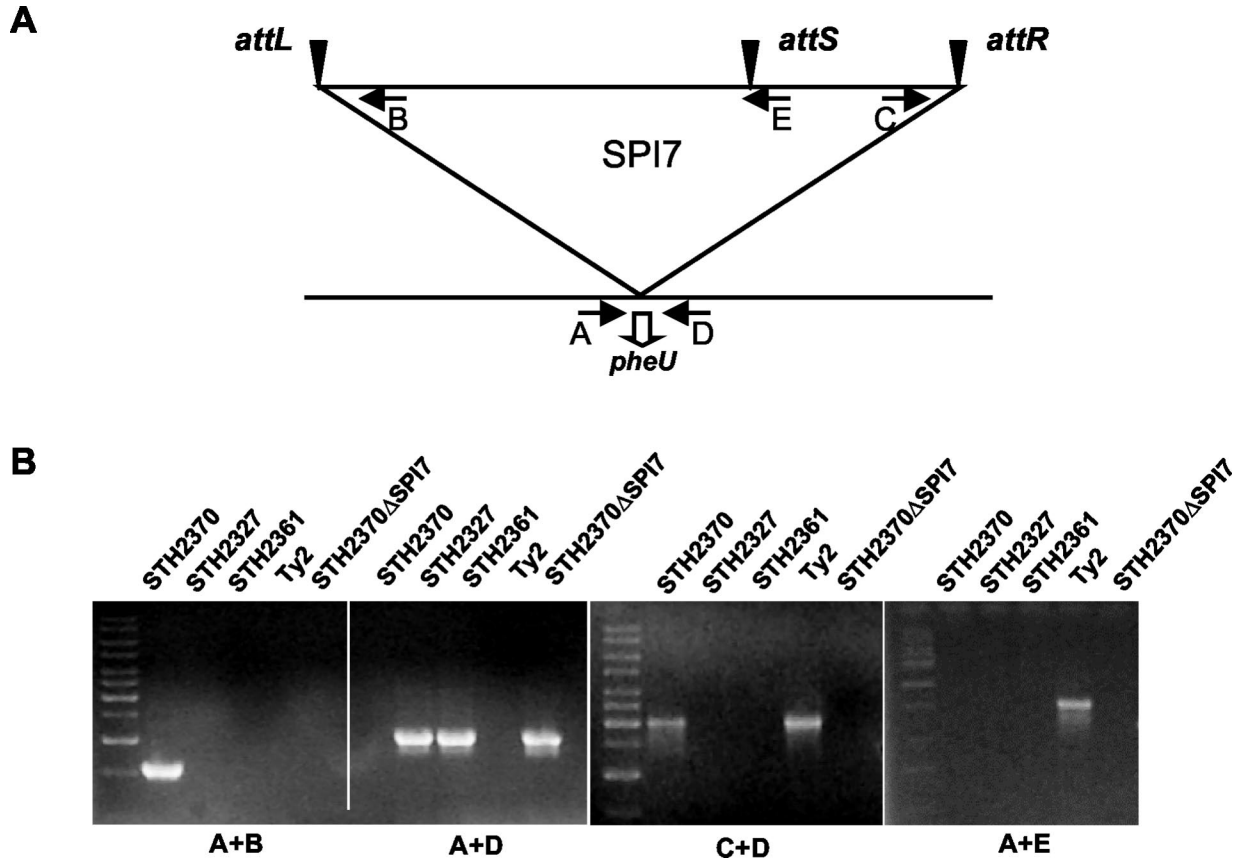

FIG. 3. Clinical isolates of *S. enterica* serovar Typhi missing a region internal to SPI7 have undergone precise deletion of SPI7. The PCR strategy for this experiment is diagrammed at the top and is described in the text. The results are shown at the bottom.

in the loss of SPI7 regions R1 and R2 and a large part of R3, including the complete *viaB* locus, and has left behind 13,117 bp at the right end of SPI7. The result that each of the seven primer pairs does not yield products with Ty2 DNA is consistent with this explanation. Because the sequence of the Ty2 genome (17) shows that it has the entire SPI7, this result prompted us to compare the genotypes of the working Ty2 strain, maintained in our laboratory, with another subclone of its source strain from the Instituto de Salud Publica, Santiago, Chile. This subclone was found to be Vi^+, suggesting that the deletion we observed in our working strain of Ty2 has occurred during the course of its routine passage in the laboratory.

We also used combinations of primers A, B, C, and D to amplify DNAs isolated from the three *S. enterica* serovar Paratyphi C strains, which we have shown have variable SPI7 structures. When genomic DNA from isolate SARB50 was used as the template, primers pair AD yielded a PCR product of the expected size for the *attB* novel join point, demonstrating that it is missing SPI7, probably due to an *attL* × *attR* recombination event; the sequence of the PCR product (GenBank accession no. AY391280) confirms this. In contrast, DNA isolated from strains SARB48 and SARB49 yielded a product of the expected size with primer pair AB but not with CD, indicating that these strains lack the *attR* boundary of SPI7 or that one or both of primers C and D do not hybridize well with these template DNAs.

Excision of SPI7 results in multiple phenotypic changes in *S. enterica* serovar Typhi. We found that STH2327, STH2361, and our working strain of Ty2 formed colonies on solid rich medium that are larger, flatter, and more opaque than those formed by the other isolates. These three strains failed to produce Vi antigen, because cell suspensions of these strains were not agglutinated by anti-Vi antibody. Because these strains fail to produce Vi antigen, they are resistant to the lytic phage Vi-II (data not shown).

This latter phenotype, resistance to phage Vi-II, provides us with a genetic enrichment for the loss of SPI7. Using this enrichment, we isolated an otherwise isogenic derivative of STH2370 with a precise deletion of SPI7, STH2370ΔSPI7. Strain STH2370 was grown to overnight density in rich medium and plated in the presence of excess phage Vi-II. Phage-resistant colonies arose with an efficiency of plating of 4×10^{-6}, and the majority of these colonies (93 of 100 tested) were not agglutinated by anti-Vi serum. PCR analysis of DNA isolated from three of these colonies confirmed that the phage-resistant strains that gave rise to these colonies have precise deletions of SPI7. Figure 4 shows the results of a comparison of the phenotype of STH2370ΔSPI7 with that of its otherwise isogenic parental strain. Like strains STH2327, STH2361, and Ty2, the colonies formed by STH2370ΔSPI7 are larger, flatter, and more opaque than those formed by its otherwise isogenic

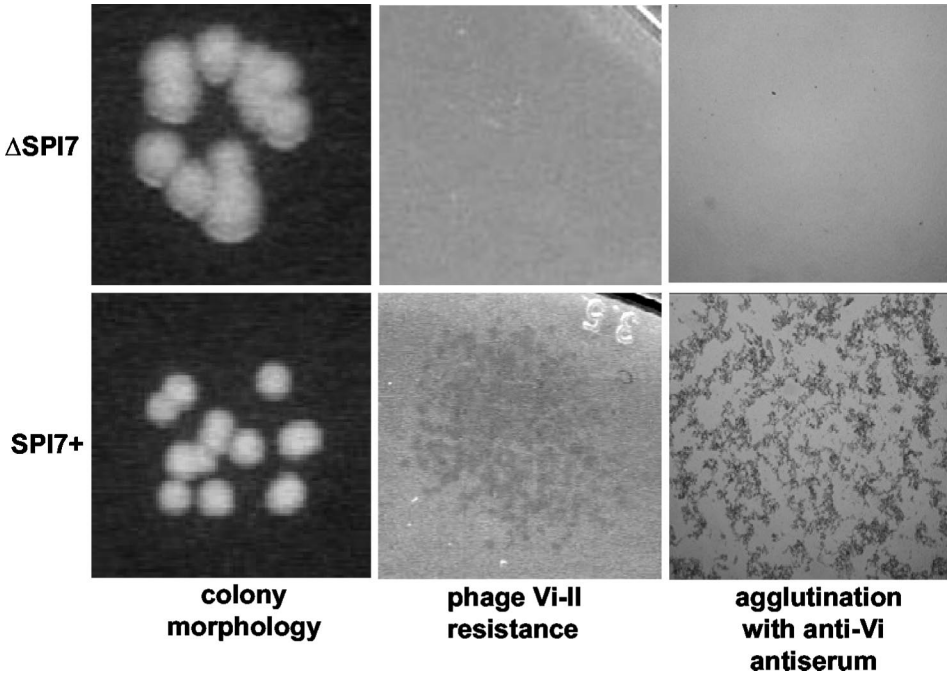

FIG. 4. Precise excision of SPI7 results in profound phenotypic changes. Shown are differences in the colony morphologies (×2 magnification) (left), sensitivities to phage Vi-II (×2 magnification) (center), and agglutination with anti-Vi antiserum (×10 magnification) (right) exhibited by otherwise isogenic derivatives of *S. enterica* serovar Typhi clinical isolate STH2370 with (SPI7+) and without (ΔSPI7) SPI7.

parent, and the mutant both fails to agglutinate with anti-Vi antibody and is resistant to phage Vi-II.

The *viaB* biosynthetic operon includes 10 genes (26), and spontaneous loss-of-function mutations in these genes can prevent the production of Vi antigen and thereby confer resistance to phage Vi-II. Therefore, phage-resistant mutants of a strain of *S. enterica* serovar Typhi with SPI7 may arise by one of two different mechanisms, either spontaneous mutation in the *viaB* operon or precise excision of SPI7. Consequently, the frequency of phage-resistant mutants will be the sum of the frequencies of mutants arising from each of these two different types of genetic event.

To determine the frequency of mutants resulting from the site-specific excision of SPI7 among phage-resistant mutants, we designed a genetic screen to distinguish between these mutants and mutants with *viaB* mutations. We replaced SPI7 region 1 (R1 in Fig. 1A) in strain STH2370 with a kanamycin resistance (Kan^r) determinant to make strain STH2370 R1::*kan*. Phage-resistant derivatives of this strain that acquire spontaneous loss-of-function mutations in the *viaB* operon will retain the Kan^r determinant and a Kan^r phenotype. In contrast, mutants that have lost the entire SPI7 due to precise excision will lose their Kan^r determinant and acquire a Kan^s phenotype.

A culture of strain STH2370 R1::*kan* was grown to overnight density in LB medium with kanamycin (to kill bacteria that potentially had lost SPI7 during growth), and an aliquot of the culture was diluted 10^8-fold into in fresh medium without kanamycin. After growth to overnight density, the culture was plated in the presence of excess phage Vi-II to select phage-resistant colonies, which arose at a frequency of 4×10^{-6}. Among these, about 1.25% were found to be Kan^s, indicating that the frequency of SPI7 excision in this strain is about 5×10^{-8} under these conditions. DNA was isolated from several Kan^s mutants following this selection and screening and amplified with primer pair AD. In all cases (14 of 14), amplification resulted in a product of the expected size arising from *attL* × *attR* recombination (data not shown). As a control for this experiment, strain STH2370 R1::*kan* was grown in parallel with kanamycin, and phage-resistant mutants were selected. Among these, 472 of 472 tested were found to be Kan^r.

We also attempted to amplify the novel join point of the circular product formed by the precise excision of SPI7 by using the primer pair BC (Fig. 3). We obtained a product of the expected size in only one of seven independent attempts (data not shown), consistent with the idea that the circular SPI7 cannot replicate and, with its measured frequency of excision, should be present at less than 0.5 copy/μg of chromosomal DNA extracted from cells grown under these conditions.

Derivatives of *S. enterica* serovar Typhi that have lost SPI7 are hyperinvasive in HEp-2 human epithelial cells. Expression of the *sopE* gene is thought to play an important role in the invasion of epithelial cells (20, 62, 67). Recent work also suggests that type IV pill are an important adhesion factor for the invasion of cultured human epithelial cells by *S. enterica* serovar Typhi (65). Therefore, we tested whether loss of SPI7 affects the ability of *S. enterica* serovar Typhi strains to invade human epithelial cells. To do this, we compared the abilities of isolate STH2370, which has SPI7, and STH2370ΔSPI7, its phage-resistant derivative missing this island, to invade human HEp-2 cells in tissue culture. Strains were grown as described in Materials and Methods and used to infect HEp-2 monolay-

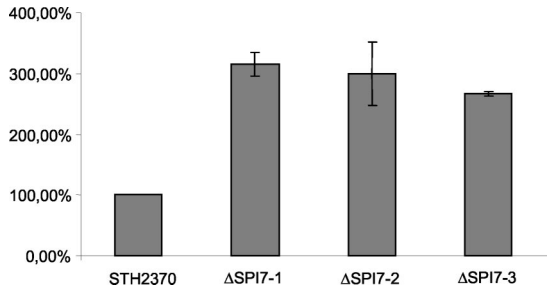

FIG. 5. Derivatives of STH2370 that have lost SPI7 are hyperinvasive in human HEp-2 cells. The titers of mutant intracellular bacteria after 1 h relative to that of the wild-type strain are a measure of the ability of each mutant to invade epithelial cells; under these conditions, about 0.2 to 0.4% of the infecting STH2370 bacteria can be recovered from human cells after gentamicin treatment. The figure shows the average results ± standard deviations from three independent determinations for three independent derivatives of STH2370 that have lost SPI7 by precise excision, as described in Materials and Methods.

ers, and invasion was measured as the percentage of bacteria that entered HEp-2 cells after 1 h of coincubation.

As shown in Fig. 5, strain STH2370ΔSPI7 was about three times more invasive than strain STH2370. These results are the opposite of those expected if the *sopE* and pilus type IV genes (both encoded within SPI7) were required for invasion of epithelial cells, demonstrating that both SPI7-encoded virulence factors are dispensable for invasion. We obtained similar results when we compared the abilities of clinical isolates STH2361 and STH2327, missing SPI7, with that of STH2370 to invade HEp-2 cells (data not shown).

DISCUSSION

The large pathogenicity island (SPI7) present in strains of *S. enterica* serovar Typhi is a 133-kb segment of DNA and has many of the genetic properties characteristic of other bacterial PIs. Most striking among these properties is that SPI7 has a G+C base composition different from that of genome in which it resides, suggesting that it has encountered the *S. enterica* serovar Typhi genome by horizontal transfer from a different serovar of *Salmonella* or from a different genus of gram-negative bacteria.

When and how did *S. enterica* serovar Typhi acquire SPI7? We have found that this island occurs only in serovars Typhi and Paratyphi C and is variable in structure in isolates of both of these serovars, lending support to the hypothesis that its acquisition is a very recent event, more recent than the speciation of *S. enterica* serovar Typhi (30, 49). Consistent with this idea, other investigations have found PIs related to SPI7 by the criterion that they carry genes involved in the synthesis of Vi antigen only in isolates of the related gram-negative bacteria *S. enterica* serovar Dublin and *Citrobacter freundii* (15, 25, 47, 57).

The stable incorporation of PIs into bacterial genomes probably involves three steps, horizontal transfer by transduction and/or conjugation, establishment by site-specific integration, and maintenance by integration per se and loss-of-function mutations that reduce the frequency of excision of PIs from the host genome (2). Several genetic features of SPI7 suggest that it may have been transferred to *S. enterica* serovar Typhi by one of two mechanisms of genetic exchange, conjugation or transduction.

The "conjugative transposons" (13) can move by conjugation between different bacterial species or genera (27, 58, 61), and SPI7 has several genes predicted to encode proteins involved in this mechanism of transfer (47). However, when applied to PIs, the term "conjugative transposon" is usually a misnomer (9). Most of these elements should not be viewed as transposons but, rather, as defective (or satellite) prophages. This is because their mechanism of integration into their host chromosomes is not that of transposition and its accompanying target site duplication but, rather, that of conservative site-specific recombination, according to the model of Campbell (10), often mediated by phage integrases (28, 51, 60). Consistent with this idea, the excision of these elements is most often precise (4, 8, 28, 39, 50, 51, 59, 60), unlike the excision of transposons, which is most often imprecise.

Because the genetic structures and properties of the vast majority of PIs resemble those of prophages in two important ways, we must consider transduction an alternative mechanism for their horizontal transfer. In particular, at least one PI is a satellite phage (53). Most PIs, including SPI7, are bounded by short, direct repeats (*att* sites) overlapping a tRNA gene (9, 22, 23) and have open reading frames (*int* genes) encoding potential integrases adjacent to an *att* site. Thus, for example, among the 47 continuous stretches of DNA larger than 1 kb that are present in the *S. enterica* serovar Typhi CT18 sequence (45) but not in the *S. enterica* serovar Typhimurium LT2 sequence (17), 8 are flanked by direct repeats of 12 bp or more and include a putative *int* gene adjacent to one of these *att* sites.

In this study, we have shown that the majority of isolates of *S. enterica* serovar Typhi missing SPI7 have resulted from recombination events that have occurred between *attL* and *attR* to generate precise deletions of SPI7 (Fig. 6). Because recombinants that have lost this region lose the ability to produce Vi antigen and become resistant to phage Vi-II, we have been able to measure the frequency of SPI7 excision to be about 5×10^{-8}/cell during the growth of *S. enterica* serovar Typhi STH2370 in the laboratory. Although this frequency of precise excision appears to be among the lowest reported for PIs (2, 28, 39, 50, 59, 60), the results of independent experiments described in the accompanying paper suggest that precise excision occurs at a much higher frequency after storage of *S. enterica* serovar Typhi strains under laboratory conditions (42). In support of this idea, we have found that our derivative of *S. enterica* serovar Typhi strain Ty2 has acquired a deletion of a large portion of SPI7 on laboratory passage. This deletion has resulted from site-specific recombination between *attL* and *attS*, a 17-bp secondary attachment site located downstream of the *viaB* operon in SPI7 (Fig. 6). Together, these results suggest that the excision of all or a large part of SPI7 is most often due to recombination mediated by a prophage-encoded integrase, a hypothesis we are now testing.

If site-specific recombination, rather than RecA-dependent homologous recombination, is the mechanism by which SPI7 deletions occur most frequently, then site-specific recombination between *attS* and *attR* should also occur during the establishment and/or maintenance of SPI7. Although the design of the experiments we report here does not address this possibil-

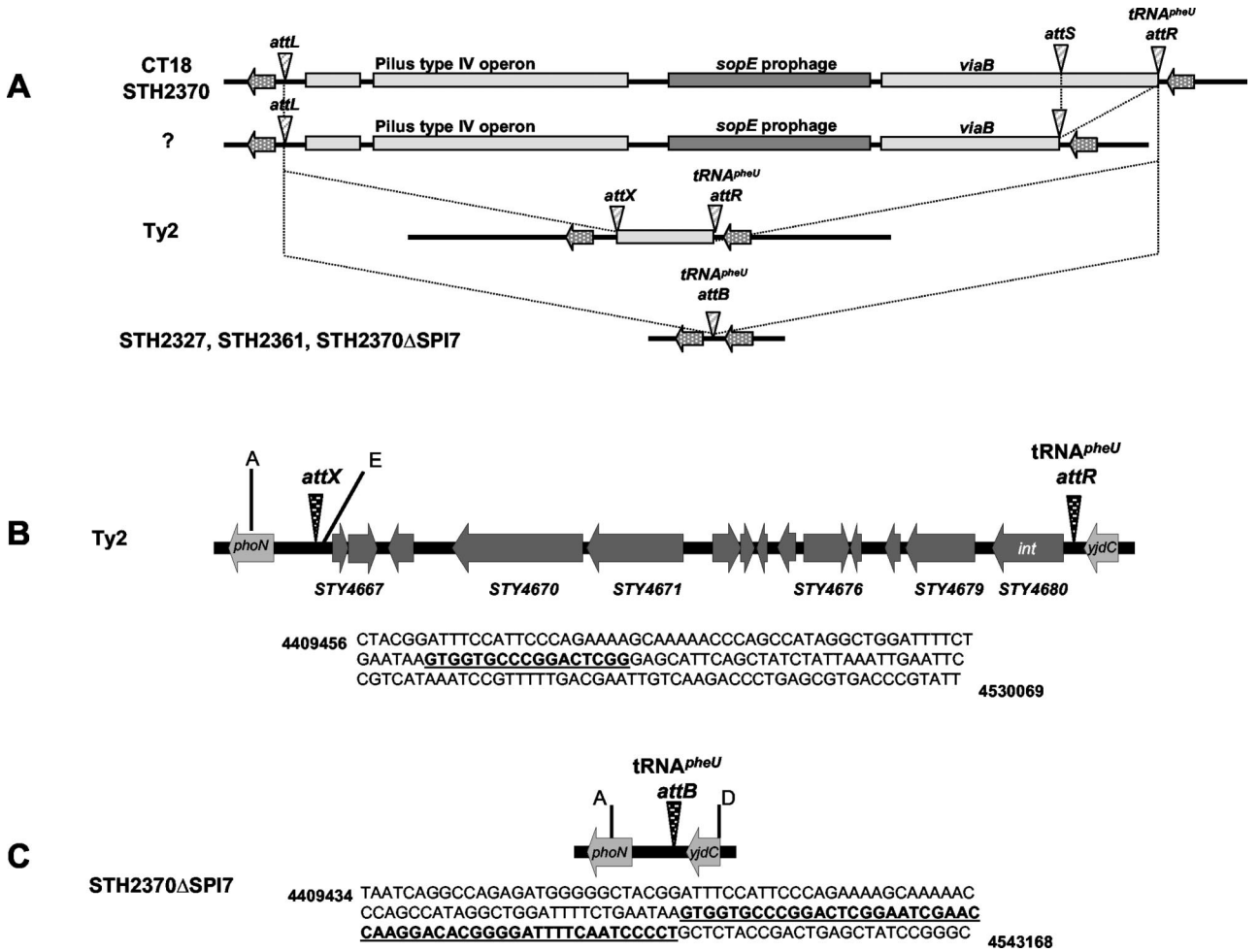

FIG. 6. Novel join points formed by site-specific recombination between the *attL*, *attS*, and *attR* sites of SPI7. (A) Structure of SPI7 found in the majority of clinical isolates of *S. enterica* serovar Typhi, including CT18 and STH2370. Although we could not detect whether *attS* × *attR* recombination could occur in strains of *S. enterica* serovar Typhi, site-specific recombination between *attL* and *attS* has occurred in our working strain of Ty2, to give rise to the novel join point, *attX*, and between *attL* and *attR* in clinical isolates STH2327 and STH2361, to give rise to the novel join point, *attB*. This is the identical join point to that found in derivatives of STH2370. (B and C) Detailed structures of the chromosomal products of site-specific recombination within SPI7 in strains Ty2 and STH2370ΔSPI7, as explained in the legend to Fig. 1, as well as the sequences of the novel join points *attX* and *attB*, respectively. Coordinates are those of the *S. enterica* serovar Typhi CT18 sequence (45).

ity for derivatives of *S. enterica* serovar Typhi, we and others found that strains of *S. enterica* serovar Paratyphi C among descendants of the SARB collection have a version of SPI7 different in structure from that found in the majority of clinical isolates of *S. enterica* serovar Typhi, because they are missing the sequence bounded by *attS* and *attR* (47).

SPI7 in *S. enterica* serovar Typhi includes no fewer than five different genes encoding potential phage integrases, STY4586, STY4645, STY4666, STY4678, and STY4680, the second of which is associated with the *sopE* cluster of genes. Therefore, it is likely to be the product of multiple events involving the repeated site-specific integration of prophages, perhaps followed by their stabilization in the host genome by recombination with other resident prophages. Events such as the latter may lead to the stable duplication of genes and may account for the fact that a second copy of the *sopE* virulence determinant resides within SPI7 (52). Consistent with this idea, SPI7, when it is found in isolates of *S. enterica* serovar Paratyphi C, appears to lack the *sopE* gene. In *S. enterica* serovar Typhi SPI7, *sopE* is embedded within an island of genes encoding homologues of phage P2 proteins. Multiple integration events, followed by intrachromosomal gene conversion events, also appear to have given rise to a subset of the variable *Escherichia coli* locus of enterocyte effacement PIs (46).

Why is SPI7 excision, most often precise and probably catalyzed by a phage integrase, such a rare event? Clues to the answer to this question come from an understanding of the biology of temperate phages. First, temperate phages control the directionality of their integration/excision reactions with their host chromosomes by at least three different mechanisms. Some phages, like phage λ, encode Xis (excisionase proteins) to alter the structure of the intasome (recombination) complex, and excision is favored over recombination only during lytic development. Others, like P2, encode Cox (control of excision) proteins to control the transcription of an integrase that catalyzes a nondirectional site-specific recombination re-

action (34). In both these paradigm cases, integration per se of the prophage results in the silencing of *int* gene expression, ultimately by reducing the level of transcription of *int* from its own promoter. In the case of phage λ, a third mechanism of "retroregulation" can also reduce the level of Int translation, in which integration reduces the stability of the *int* mRNA by changing the sequence of its 3′ terminus (21).

Indeed, after integrating as prophages, the majority of temperate phages appear to favor lysogenic over lytic development by reducing the relative frequency of excision versus integration due to the integration event per se. The results of microarray experiments show that among the four resident prophages in *S. enterica* serovar Typhimurium LT2 (Fels-1, Gifsy-1, Gifsy-2, and Fels-2) induced in response to DNA damage, only one, Fels-2, can excise from the host genome efficiently (48). Induction of the other three prophages results in the amplification not only of the prophage genomes but also of adjacent *S. enterica* serovar Typhimurium DNA, despite the fact that all four prophage genomes have homologues of *xis* genes.

Nonetheless, such prophages can excise precisely from their host genomes spontaneously, although this occurs only rarely. Presumably the occasional higher-level expression of their *int* genes in individual cells among large populations of lysogenic cells may accompany prophage induction, as is the case for P2, and supernatants of liquid cultures of lysogens have plaque-forming phages. Even the rare release of plaque-forming phages has profound consequences. Cocultures of a nonlysogenic *S. enterica* serovar Typhimurium strain with a lysogenic strain results in the rapid lysogenic conversion of the nonlysogenic strain, imposing a strong selection for the maintenance and spread of integrated prophage genomes (5) and perhaps their satellite PIs.

Like other PIs, SPI7 contains genes encoding known pathogenicity determinants, including *sopE* (24) and an operon involved in the synthesis of type IV pili (65). Both of these determinants play an important role in the invasion of mammalian cells by *S. enterica* serovar Dublin (41, 62), and *sopE2* is required for the efficient invasion of host epithelial cells by *S. enterica* serovar Typhimurium (3). However, we found that otherwise isogenic derivatives of a clinical isolate missing SPI7, as well as independent clinical isolates missing SPI7, are hyperinvasive in human epithelial cells in tissue culture, the opposite of what we would predict if the *sopE* and type IV pili genes were required for the invasion of epithelial cells. Presumably, the second copy of the *sopE* gene (*sopE2*) present in the *S. enterica* serovar Typhi genome (STY1987) is sufficient to promote the invasion of epithelial cells in the absence of SPI7-encoded SopE function. Our results are consistent with those of other studies showing that Vi-negative strains of *S. enterica* serovar Typhi are hyperinvasive in epithelial cells (40) and more destructive of Peyer's patches (66); this is probably because the expression of many SPI7 genes is controlled by the osmolarity-responsive RscB-RscC two-component regulatory system. Under high-osmolarity conditions, expression of the Vi antigen from SPI7 is repressed, and both the secretion of Sips and flagellar assembly, necessary for invasion, are activated. Under low-osmolarity conditions, the opposite is the case. Thus, there appears to be a competition between the expression of Vi antigen and type IV pilus genes and the expression of other genes required for virulence (1).

Why, then, do the majority of clinical isolates of *S. enterica* serovar Typhi have SPI7? We note that *sopE2* is annotated as a pseudogene (with a frameshift mutation) in the *S. enterica* serovar Typhi CT18 genome sequence (45) and may be naturally variable among independent isolates of *S. enterica* serovar Typhi. This frameshift mutation is not found in the genome sequence of laboratory strain Ty2 (17). Thus, in some, but not all, isolates of *S. enterica* serovar Typhi, SPI7-encoded virulence determinants may be essential for the successful systemic infection of the human host. Alternatively, it is possible that SPI7 plays a role not in the virulence of *S. enterica* serovar Typhi but in its transmission. Recent outbreaks of *S. enterica* serovar Typhi in India have involved strains that have lost Vi antigen expression (38), suggesting that the *viaB* locus is not required for the virulence of *S. enterica* serovar Typhi and that perhaps SPI7 has a different selective advantage.

The finding that a significant fraction of our nosocomial isolates of *S. enterica* serovar Typhi are missing SPI7 supports this idea and is troubling. Currently, the available vaccine against *S. enterica* serovar Typhi has been prepared against purified Vi antigen and is not be effective against emerging Vi-minus strains. Thus, the facts that a significant fraction of natural isolates of *S. enterica* serovar Typhi may be Vi negative and that SPI7 can undergo precise excision in Vi-positive clinical isolates to generate virulent Vi-negative derivatives may account for the finding that the vaccine has had only a limited (55%) efficacy in areas of endemic infection areas (32). We suggest that a more effective vaccine against *S. enterica* serovar Typhi should be based on a combination of Vi antigen and another antigen presented by Vi-negative strains.

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