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Salmonella typhi Contains Identical Intervening Sequences in All Seven *rrl* Genes

NEIL R. MATTATALL,* DAYLE A. DAINES,† SHU-LIN LIU, AND KENNETH E. SANDERSON

Salmonella Genetic Stock Centre, Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4

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***Salmonella typhi* Ty2 *rrl* genes contain intervening sequences (IVSs) in helix-25 but not in helix-45 on the basis of observed 23S rRNA fragmentation caused by IVS excision. We have confirmed this and shown all seven IVSs to be identical by isolating genomic DNA fragments containing each of the seven *rrl* genes from *S. typhi* Ty2 by use of pulsed-field gel electrophoresis; each *rrl* gene was amplified by PCR in the helix-25 and helix-45 regions and cycle sequenced. Thirty independent wild-type *S. typhi* strains, tested by genomic PCR and *Dra*I restriction, also have seven *rrl* genes with helix-25 IVSs and no helix-45 IVSs. We propose that IVS homogeneity in *S. typhi* occurs because gene conversion drives IVS sequence maintenance and because adaptation to human hosts results in limited clonal diversity.**

Several *Salmonella* species (considered serovars by some authors) possess intervening sequences (IVSs) of 90 to 110 bp in some or all of the seven *rrl* (23S rRNA) genes at base pair 550 and base pair 1170 (*Escherichia coli rrl* numbering [15]) (3, 8, 9, 14, 19, 20). These sites correspond to helix-25 and helix-45, respectively, in the proposed secondary structure of the *E. coli* 23S rRNA. IVSs replace these small helices, in part, with an extended stem and loop. Following *rrn* operon transcription, IVSs are cut by RNase III in the stem region, and this cutting causes 23S rRNA to fragment into two or more pieces without any apparent loss in functionality (3).

Salmonella typhimurium LT2 possesses three unique IVSs: two are extensions of helix-25, and one is an extension of helix-45 (14). The two helix-25 IVSs occur in *rrlG* and *rrlH* and are only 56% identical, while the helix-45 IVS is 100% identical in *rrlA*, *rrlB*, *rrlC*, *rrlD*, *rrlE*, and *rrlH*. This distribution of IVSs among the seven *rrl* genes determines 23S rRNA fragmentation stoichiometry (see Fig. 1). The *rrlH* helix-25 IVS was 94% identical to the nucleotide sequence of the helix-25 IVS from *Salmonella arizonae* (3). Similarly, the helix-45 IVS was 100% identical to IVSs from two other *S. typhimurium* strains (3, 19) and was strongly related to IVSs from *S. arizonae* (3) and *Yersinia enterocolitica* (19). We have suggested that these three unique IVSs entered *S. typhimurium* LT2 through lateral transfer and that the helix-45 IVS was distributed to and maintained in the same sequence in six of the seven *rrl* genes through the mechanism of gene conversion (14).

Salmonella typhi is the only *Salmonella* species that grows exclusively in humans and causes typhoid enteric fever. Many studies have shown that independent *S. typhi* strains from different geographic regions are very similar, indicating that their origin is clonal in nature, with no significant recombination with other *Salmonella* species and limited divergence by mutation. For example, by using multilocus enzyme electrophoresis, Reeves et al. (16) showed that *S. typhi* strains constitute a single clone, while Selander et al. (18) found two clones, but both groups propose that *S. typhi* is a relatively homogeneous group widely separated from the other species in *Salmonella* subgenus I. Envelope protein profiles also reveal homogeneity of strains (5). The objective of this report is to examine the homogeneity of IVS possession and nucleotide sequence in *S. typhi*.

23S rRNA fragmentation in *S. typhi* Ty2. *S. typhi* Ty2 line H251.1 (which is derived from wild-type strain Ty2 and which carries an *aroC* deletion that reduces pathogenicity [7]) does not contain intact 23S rRNA (2.9 kb) as detected for *E. coli* K-12; instead, 2.4- and 0.5-kb rRNA fragments (in addition to 16S rRNA) were observed (Fig. 1A). This fragmentation pattern is consistent with the presence of helix-25 IVSs in all seven *rrl* genes, since RNase III cutting at the helix-25 site would generate the fragments observed (Fig. 1B). *S. typhi* Ty2 RNA does not contain 1.7-, 1.2-, or 0.7-kb fragments, which supports the conclusion that *S. typhi* Ty2 does not possess helix-45 IVSs; fragments of these sizes are seen in *S. typhimurium* RNA, as expected (Fig. 1) (14).

Isolation and sequencing of the seven *rrl* genes of *S. typhi* Ty2. *S. typhi* Ty2 *rrl* genes were separated by pulsed-field gel electrophoresis (PFGE) on the basis of the genomic cleavage map for *S. typhi* Ty2 (13) by methods described previously (12). Genomic fragments digested with I-*Ceu*I (New England Biolabs) allowed for isolation of *rrlA*, *rrlB*, *rrlE*, *rrlG*, and *rrlH*; *rrlC* and *rrlD* were copurified on a single I-*Ceu*I fragment that was excised in agarose, restricted with *Spe*I (Boehringer Mannheim), and electrophoresed (by PFGE) to yield two fragments, each containing one *rrl* gene (Table 1). All fragments were purified by GlassMAX (GIBCO BRL) and used as a template for PCR to generate amplicons A and B for each *rrl* gene (Fig. 2). PCRs were performed on a Techne Gene "E" thermal cycler under conditions recommended by the *Taq* polymerase supplier (GIBCO BRL). Agarose gel electrophoresis of amplicon A from all seven *S. typhi* Ty2 *rrl* genes showed that all amplicons were the same size, about 110 bp larger than the amplicon from *E. coli* whole genomic template (data not shown). This indicates that all *S. typhi* Ty2 *rrl* genes contain helix-25 IVSs, as predicted by the RNA fragmentation data (Fig. 1A). Amplicon B from all *rrl* genes was the same size as amplicon B from *E. coli* (data not shown), indicating, as predicted by RNA data (Fig. 1A), that none of the *rrl* genes of *S. typhi* Ty2 possesses helix-45 IVSs.

* Corresponding author. Phone: (403) 220-3572. Fax: (403) 289-9311. Electronic mail address: nmattata@acs.ucalgary.ca.

† Present address: Department of Microbiology and Immunology, University of Rochester Medical Centre, Rochester, NY 14642.

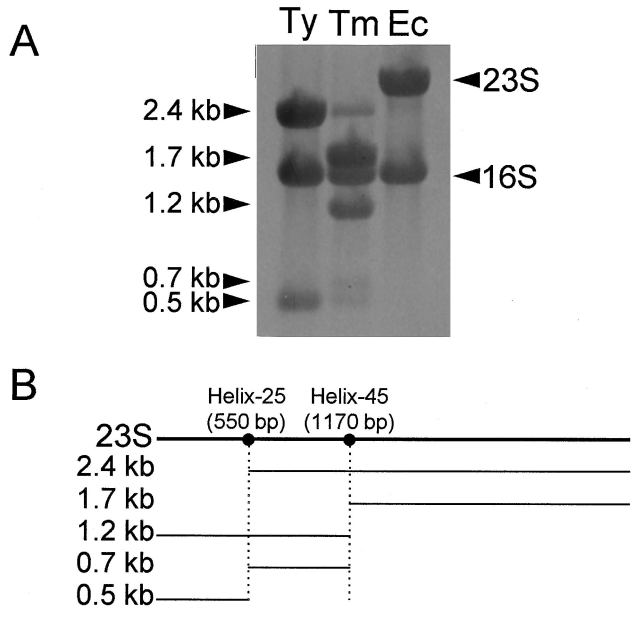

FIG. 1. (A) 23S rRNA fragmentation in *S. typhi* Ty2 (lane Ty) was observed by isolation of RNA by the hot phenol method as reported previously (14), followed by glyoxal-dimethyl sulfoxide–1.2% agarose gel electrophoresis of 10 μg of the RNA, blotting to Hybond-N$^+$ (Amersham) as recommended, and staining with methylene blue (17). 23S rRNA from *S. typhi* Ty2 produced 2.4-kb and 0.5-kb fragments. The results with *S. typhimurium* LT2 (lane Tm) indicate the positions of all possible 23S rRNA fragments; *S. typhimurium* LT2 contains one of the seven *rrl* genes with an IVS in helix-25 only (2.4- and 0.5-kb fragments), five with an IVS in helix-45 only (1.7- and 1.2-kb fragments), and one that has IVSs in both helices (1.7-, 0.7-, and 0.5-kb fragments) (14). The results with *E. coli* K-12 (lane Ec) reveal only 23S and 16S rRNA. (B) 23S rRNA fragmentation schematic. 23S rRNA fragments of 2.4 and 0.5 kb indicate that one *rrl* gene carries an IVS in helix-25 (about base pair 550 in *rrl*). 23S rRNA fragments of 1.7 and 1.2 kb indicate that one *rrl* gene carries an IVS in helix-45 (about base pair 1170 in *rrl*). 23S rRNA fragments of 1.7, 0.7, and 0.5 kb indicate that one *rrl* gene carries IVSs in both helices.

Both amplicons A and B were gel purified and cycle sequenced at the University Core DNA Lab, University of Calgary, by using the forward and reverse primers 5′TACTCCT GACTGACCGATAG3′ and 5′GGCTAGATCACCGGGTT TCG3′ for amplicon A and 5′CCTGCGCGGAAGATGTAA CG3′ and 5′GCATTCGCACTTCTGATACC3′ for amplicon

TABLE 1. Genomic cleavage fragments containing *S. typhi* Ty2 *rrl* genes[a]

rrl gene locus[b]	Cleavage enzyme	Fragment size (kb)
A	I-*Ceu*I	146
B	I-*Ceu*I	44
C[c]	I-*Ceu*I/*Spe*I	159
D[c]	I-*Ceu*I/*Spe*I	343
E	I-*Ceu*I	136
G	I-*Ceu*I	828
H	I-*Ceu*I	724

[a] Separation of fragments by PFGE was done as described previously (11). The data are from the *S. typhi* Ty2 genomic cleavage map (13).

[b] Although each *rrl* gene is denoted by a single letter, the *S. typhi* Ty2 genomic cleavage map (13) designates some of the genes as hybrids to represent postulated genomic recombination events. We have substituted the hybrid *rrl* gene letters with single gene letters corresponding to their order on the *S. typhimurium* LT2 genome (10).

[c] *rrlC* and *rrlD* were copurified on the I-*Ceu*I 502-kb fragment. This fragment was restricted at a single *Spe*I site and separated by PFGE into the fragment sizes shown.

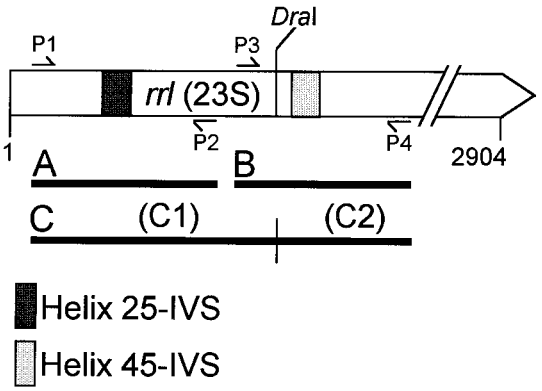

FIG. 2. PCRs were performed with the following primers manufactured by the University Core DNA Lab (University of Calgary): P1 (5′GCGTCGGTAA GGTGATATG3′), P2 (5′GCTATCTCCCGGTTTGATTG3′), P3 (5′CCGATG CAAACTGCGAATAC3′), and P4 (5′TTCTCTACCTGACCACCTG3′). These primers locate to *E. coli rrlB* bases 74 to 92, 786 to 805, 901 to 920, and 1616 to 1634, respectively (15). Amplicon A used primers P1 and P2; amplicon B used primers P3 and P4; amplicon C used primers P1 and P4. Amplicons A (731 bp without IVS, according to *rrnB* gene sequence [15]) and B (733 bp without IVS) were sequencing templates for nested primers in the helix-25 and helix-45 regions, respectively. Amplicon C was used to detect total genomic IVS possession. Restricting amplicon C with *Dra*I yielded two fragments from each *rrl* gene: the C1 fragment (1008 bp without IVS) reported on the basis of the presence of the helix-25 IVS (~110 bp) and the C2 fragment (551 bp without IVS) reported on the basis of the presence of the helix-45 IVS (~90 bp). Therefore, the genomic distribution of IVSs among the seven *rrl* genes was determined from length and intensity variance of four fragments.

B. All seven amplicon A (helix-25 IVS) and amplicon B (helix-45) products were found to be 100% identical (Fig. 3). The *S. typhi* Ty2 helix-25 IVS was folded into RNA secondary structure by use of FoldRNA (22) and then visualized with LOOPVIEWER (6). The resultant fold was similar in proposed secondary structure to the helix-25 IVS from *rrlH* of *S. typhimurium* LT2 (Fig. 3). When the nucleotide sequence from the *S. typhi* Ty2 helix-25 IVS was compared with that of other species by use of the Genetics Computer Group GAP program, the helix-25 IVS from *rrlH* of *S. typhimurium* LT2 was 97% identical and the helix-25 IVS from *S. arizonae* was 94% identical (Table 2). In contrast, the *S. typhimurium* LT2 *rrlG* helix-25 IVS was very different in secondary structure and only 55% identical to the *S. typhi* helix-25 IVS (Fig. 3; Table 2). The helix-45 pentaloop was identical in all seven *rrl* genes from *S. typhi* Ty2 but differed from the *S. typhimurium* LT2 *rrlG* tetraloop by several base changes and one nucleotide deletion in the cap (Fig. 3).

Distribution of IVSs among wild-type *S. typhi* strains. To determine IVS possession in *S. typhi*, whole genomic DNA from 35 strains representing 30 independent wild-type strains (Table 3) was amplified by PCR to produce amplicon C (Fig. 2). Amplicon C was restricted by *Dra*I to produce the C1 fragment containing the helix-25 region and the C2 fragment containing the helix-45 region; the amplicons were separated by agarose gel electrophoresis. All *Dra*I restriction patterns were the same for all 35 strains; eight representative strains are shown in Fig. 4 (lanes A to H). These data indicate that all strains possess helix-25 IVSs in all seven *rrl* genes and helix-45 in none, as shown earlier for strain Ty2 (Fig. 4, lane E). The *rrnB* operon from randomly selected *S. typhi* strains 9032-85 and IP E.88.374 (Table 3) was isolated by PFGE and amplified by PCR, and the nucleotide sequence of the IVS was found to be 100% identical to the Ty2 helix-25 IVS, confirming the homogeneity of IVSs in *S. typhi*.

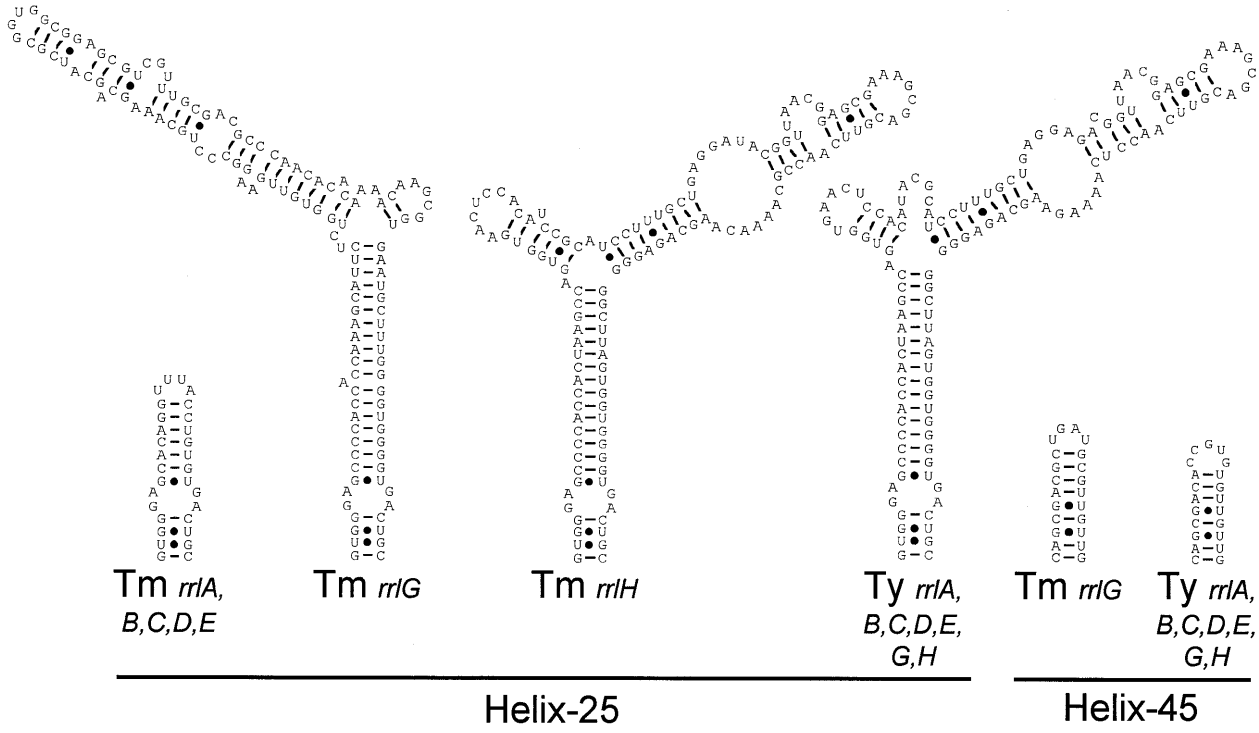

FIG. 3. Potential RNA secondary structure for IVSs and loop regions. The secondary structure of *S. typhi* Ty2 (Ty) helix-25 IVS (free energy, −49.8 kcal/mol [ca. −208 kJ/mol]) resembles that of *S. typhimurium* (Tm) *rrlH*. RNA folds for helix-25 (with and without IVSs) and helix-45 from *S. typhimurium* LT2 (Tm) are shown for comparison (14). GenBank accession numbers are as follows: *rrlA*, U54692; *rrlB*, U54693; *rrlC*, U54694; *rrlD*, U54695; *rrlE*, U54696; *rrlG*, U54697; *rrlH*, U54698.

Gene conversion and evolution. The 100% sequence identity of all helix-25 IVSs in all seven *rrl* genes of strain Ty2 and in the one *rrl* gene sequenced in each of two other *S. typhi* wild-type strains strongly supports gene conversion as being responsible for the chromosomal distribution and sequence maintenance of IVSs. With regard to distribution, a laterally transferred *rrl* gene containing an IVS may have recombined with *S. typhi* early in its evolution; this IVS-containing *rrl* gene could serve as a template for gene conversion of the other six *rrl* genes by use of a double-stranded-break repair mechanism (21). Once multiple IVS-containing *rrl* genes have been established, continued conversion could maintain IVS sequence identity in spite of a mutation that would tend to cause sequence variation. In this way, gene conversion probably operates on *rrn* operons in general to maintain sequence fidelity, as all *S. typhi* Ty2 helix-45 pentaloop sequences were identical to each other but different from the sequences in *S. typhimurium* LT2 (Fig. 3) and *E. coli* K-12 (14). If IVSs are distributed and maintained by gene conversion, they would not be required to confer a selective advantage on their host; although they must be at least selectively neutral, and this may result from their excision by RNase III. The IVS sequence variation between different species in Table 2 can therefore be explained by mutation. When an IVS has been initially transferred to a species and is present in only a single copy, random mutations that do not disrupt the RNase III site could occur and a new mutant IVS sequence could be established by subsequent gene conversion.

In contrast to the IVS homogeneity observed in *S. typhi*, 21 different wild-type strains of *S. typhimurium* from Salmonella Reference Collection A (1) fall into four different groups that possess different numbers of IVSs in their *rrl* genes (14). We

TABLE 2. Comparison of *S. typhi* Ty2 helix-25 IVS with other helix-25 IVSs[a]

Helix-25 IVS source	% Nucleotide sequence identity with helix-25 IVS of:			
	S. arizonae	*S. typhimurium* LT2 *rrlG*	*S. typhimurium* LT2 *rrlH*	*S. typhi* Ty2
S. arizonae				
S. typhimurium LT2 *rrlG*	56			
S. typhimurium LT2 *rrlH*	94	56		
S. typhi Ty2	94	55	97	

[a] The Genetics Computer Group GAP program was used to generate identity comparisons.

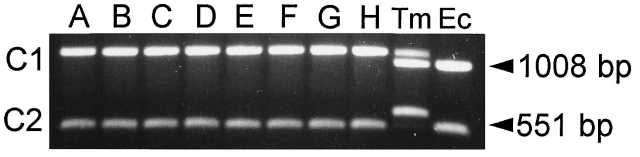

FIG. 4. Amplicons detected following PCR with whole genomic DNA. DNA from 35 *S. typhi* strains (Table 3), isolated from embedded agarose blocks (11) with GlassMAX, was amplified to amplicon C as described previously (14). Each amplicon was purified and quantitated by use of a spectrophotometer, and 600 ng was restricted with *Dra*I; the resultant DNA was electrophoresed in 1% agarose and stained with 0.5 μg of ethidium bromide per ml. Lanes A (strain 1196-74), B (1707-81), C (382-82), D (9228-77), E (H251.1), F (IP E.88.374), G (IP E.88.353), and H (ST168) contain representatives of the 35 strains, all of which gave indistinguishable results. Genomic DNA of *S. typhimurium* LT2 (lane Tm) gave a heterogeneous distribution of fragments, as expected on the basis of its known IVS possession (14). PCR of genomic DNA of *E. coli* K-12 (lane Ec) yielded the C1 and C2 fragments, calculated to be 1008 and 551 bp, respectively, on the basis of the *E. coli rrlB* gene sequence (15).

TABLE 3. *S. typhi* strains used as source of whole genomic DNA to produce amplicon C by PCR

Strain designation	Origin	Source or reference[a]
1196-74	Mexico	16
1707-81	Liberia	16
25T-35	Canada	R. Khakhria, LCDC
25T-36	Canada	R. Khakhria, LCDC
25T-37	Canada	R. Khakhria, LCDC
25T-38	Canada	R. Khakhria, LCDC
25T-39	Canada	R. Khakhria, LCDC
25T-40	Canada	R. Khakhria, LCDC
25T-41	Canada	R. Khakhria, LCDC
3137-78	India	16
3815-73	Unknown	16
383-82	Marshal Islands	16
415Ty	Netherlands	4
417Ty	New Caledonia	4
9032-85	Taiwan	16
9228-77	El Salvador	16
H238.1[b]	Chile	7
H251.1[c,d]	USSR[e]	7
In14	Indonesia	T. Pang
In15	Indonesia	T. Pang
IP E.88.353	Dakar	2
IP E.88.374	Dakar	2
ISP-1820	Chile	7
SA4828	Canada	C. Anand, Alberta Provincial Lab
ST1002	Malaysia	T. Pang
ST143	Malaysia	T. Pang
ST145	Malaysia	T. Pang
ST168	Malaysia	T. Pang
ST24	Malaysia	T. Pang
ST495	Malaysia	T. Pang
ST60	Malaysia	T. Pang
Ty21a[d]	USSR	B. Stocker
Ty2[d]	USSR	7
Ty2 LCDC[d]	USSR	R. Khakhria, LCDC
Ty514[d]	USSR	4

[a] LCDC, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada.
[b] Strain H238.1 is *aroC1019* and derived from ISP-1820.
[c] Strain H251.1 is *aroC1019* and derived from Ty2.
[d] Strains are isolates of the wild-type strain Ty2.
[e] USSR, countries of the former Soviet Union.

postulate that IVS homogeneity in *S. typhi* may occur because restriction to growth in humans limits occupation of different ecological niches and consequently reduces genetic variability by selecting for a clonal population; this is unlike *S. typhimurium*, which grows in many different hosts.

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