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Salmonella typhimurium LT2 Possesses Three Distinct 23S rRNA Intervening Sequences

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The *rrl* genes for 23S rRNA of *Salmonella typhimurium* LT2 are known to carry intervening sequences (IVSs) at two sites, helix-25 and helix-45, which are excised by RNase III during rRNA maturation, resulting in rRNA which is fragmented but nevertheless functional. We isolated DNA fragments containing the seven *rrl* genes from *Bln*I, I-*Ceu*I, and *Spe*I genomic digests following pulsed-field gel electrophoresis and used these DNA fragments as templates for PCRs utilizing primers upstream and downstream of helix-25 and helix-45. Variance in amplicon length and cycle sequencing indicated that *rrlG* and *rrlH* have IVSs in helix-25 of ~110 bp which are only 56% identical. *rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, and *rrnH* have IVSs of ~90 bp in helix-45, and all have the same nucleotide sequence. Twenty-one independent wild-type strains of *S. typhimurium* from Salmonella Reference Collection A were analyzed for IVSs by using PCRs with genomic DNAs and by denaturing agarose electrophoresis of RNAs. Many strains resemble LT2, but some have no IVSs in helix-25 and others have IVSs in helix-45 in all seven *rrl* genes. However, the IVSs in individual wild-type lines are relatively stable, for several LT2 isolates separated over many years by many single-colony isolations are indistinguishable from one another, with the exception of line LB5010, which differs by one helix-25 IVS. We postulate that IVSs have entered strain LT2 by three independent lateral-transfer events and that the IVS in helix-45 was dispersed to and maintained in the same sequence in six of the seven *rrl* genes by the mechanism of gene conversion.

The prokaryotic 50S ribosomal subunit contains contiguous 23S and 5S rRNAs; in certain genera of bacteria, such as *Campylobacter* (14, 19, 32), *Leptospira* (11, 25, 26), *Rhodobacter* (15, 17), *Salmonella* (4, 12, 13, 29, 30, 33), and *Yersinia* (29), the 23S rRNA can be fragmented into two or more pieces. In *Salmonella typhimurium* LT2, 23S rRNA fragmentation is caused by RNase III excision (without repair) of novel intervening sequences (IVSs) of ~90 to 110 bp (4). Most IVSs do not contain open reading frames or terminal consensus sequences to facilitate any other type of excision or mobilization. For this reason, IVSs are not true introns but are related elements that disrupt the normal continuity of a gene without affecting its function. The 23S rRNA fragments maintain functionality, presumably through secondary structure and ribosomal protein interactions in the 50S subunit.

S. typhimurium possesses two distinct types of IVSs, on the basis of rRNA fragment stoichiometry (4), one at about bp 550 and another at about bp 1170 in the *rrl* gene (for the 23S rRNA) (*Escherichia coli* gene numbering [23]). These positions correspond to helix-25 and helix-45 in the postulated secondary structure of the 23S rRNA of *E. coli*; both of these helices represent small tetraloops. IVSs partly replace these small helices with an extended helix and loop. Sequencing of two helix-45 IVSs isolated from two independent strains of *S. typhimurium*, ATCC 23566 (4) and 13311 (29), revealed that they are identical. Helix-25 IVSs were postulated to exist among the seven *rrl* genes of strain ATCC 23566 but were not isolated. However, both IVS types from *S. arizonae* have been sequenced (4). The helix-45 IVSs of *S. arizonae* and *S. typhimurium* have similar nucleotide sequences, suggesting a common ancestry. Further, a comparison of the helix-45 IVSs of *Yersinia enterocolitica* group 2 and *S. typhimurium* showed strong identity in a background of low chromosomal homology (29). This suggests that IVSs are recent evolutionary additions to *rrl* that have been introduced through lateral transfer, conceivably by plasmid-mediated conjugation or phage-mediated transduction.

Up to now, no one has determined how many and which of the specific *rrl* genes of *S. typhimurium* have IVSs in helix-25 or helix-45 or determined their stability. To do this, we used the genetically analyzed strain *S. typhimurium* LT2 and isolated *rrl* genes from *Bln*I, I-*Ceu*I, and *Spe*I genomic digests following pulsed-field gel electrophoresis (PFGE) by using methods described earlier (21). We found that five of the seven *rrl* genes of strain LT2 have IVSs in helix-45 only, one has an IVS in helix-25 only, and one has IVSs in both. Independent wild-type strains of *S. typhimurium* differ greatly in IVS content. However, isolates of strain LT2 separated for a period of greater than 30 years are unaltered in IVS content, with the exception of line LB5010, which lacks one helix-25 IVS.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. All of the *S. typhimurium* strains used are available from the Salmonella Genetic Stock Centre (SGSC). Reference wild-type strain LT2 is from a set of 22 *S. typhimurium* strains isolated by Lilleengen (18); this isolate has been substituted for SARA2 (strain LT2), which was part of the Salmonella Reference Collection A (SARA) set obtained from R. K. Selander (2). All strains were grown on Luria-Bertani medium (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 3.5 ml of 1 M NaOH per liter) at 37°C; Luria-Bertani agar also contained 1.5% agar (Difco). Strains were maintained in 15% glycerol at −70°C, and a single colony was isolated prior to use.

Enzymes and chemicals. Endonucleases were obtained from New England Biolabs (*Bln*I and I-*Ceu*I), Pharmacia (*Dra*I), and Boehringer Mannheim (*Spe*I). *Taq* polymerase was obtained from GIBCO BRL, and deoxynucleoside triphosphates were from Pharmacia. DNase I was from Pharmacia, and rRNasin was from Promega. Most other chemicals, including agarose, were from Sigma Chemical Co.

***S. typhimurium* LT2 *rrl* DNA template isolation from PFGE.** Preparation of high-molecular-weight genomic DNA, endonuclease cleavage of DNA in agarose blocks, and separation of DNA fragments by PFGE were done as previously

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TABLE 1. Genomic cleavage fragments containing *S. typhimurium* LT2 *rrl* genes[a]

rrl gene locus	Cleavage enzyme	Fragment size (kb)
A[b]	I-*Ceu*I	92
B[b]	I-*Ceu*I	145
C[b]	*Bln*I	90
D[c]	*Spe*I	407
E[b]	I-*Ceu*I	44
G[c]	*Spe*I	226
H[b,d]	*Bln*I	1,580

[a] Separation of fragments by PFGE was done as previously described (23).
[b] From *S. typhimurium* LT2 genomic cleavage map for *Bln*I and I-*Ceu*I (23).
[c] From *S. typhimurium* LT2 genomic cleavage map for *Spe*I (22).
[d] Note that the 1,580-kb *Bln*I fragment contains both *rrlE* and *rrlH*; however, a partial *rrl* gene of *rrnH* was retrieved through the use of amplicon D (Fig. 1).

reported (21). Fragments containing specific *rrl* genes were chosen on the basis of a genomic cleavage map for *Bln*I, I-*Ceu*I (21), and *Spe*I (20). In Table 1, the endonucleases used and fragment sizes for the isolation of *S. typhimurium* LT2 *rrl* genes are indicated. Each fragment was excised in agarose under longwave UV light and purified from the agarose by using GlassMAX (GIBCO BRL) in accordance with the manufacturer's instructions.

Genomic DNA isolation. The cells were grown overnight with shaking in Luria-Bertani broth, 5 ml of culture was centrifuged at 5,000 × *g* for 15 min, the pellet of cells was resuspended in 400 μl of proteinase digestion buffer (Tris-Cl at 200 mM, EDTA at 25 mM, NaCl at 0.3 M [pH 8.0]), and 100 μl of 10% sodium dodecyl sulfate was added. Proteinase K was then added to a final concentration of 100 μg/ml, and the solution was incubated at 42°C until it cleared (usually 1 h). A 500-μl volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the solution was vortexed and centrifuged; this step was repeated twice more with the aqueous phase. DNase-free RNase was added to a final concentration of 20 μg/ml, and the tube was incubated for 30 min at 37°C. The genomic DNA was then extracted with an equal volume of chloroform and precipitated with 2.5 volumes of ethanol, washed with 70% ethanol, and resuspended in 500 μl of H_2O.

Oligonucleotide primers. PCRs were performed with the following primers from University Core DNA Services (Health Science Centre, University of Calgary): P1 (5′gcgtcggtaaggtgatatg3′), P2 (5′gctatctcccggtttgattg3′), P3 (5′ccgatgcaaactgcgaatac3′), and P4 (5′ttctctacctgaccacctg3′). These primers were located at *E. coli rrlB* bases 74 to 92, 786 to 805, 901 to 920, and 1616 to 1634, respectively (23). Primer P5 (5′ggctgtcgtcagctcgtgt3′) was located at *E. coli rrsB* bases 1056 to 1074 (23).

PCR protocol. Refer to Fig. 1 for amplicon identification. PCRs were carried out in accordance with the instructions accompanying the *Taq* polymerase, on a Techne Gene E thermal cycler. PFGE fragment templates were titrated from GlassMAX preparations to optimize amplification, while genomic PCR was done with 100 ng of genomic DNA purified as described above. Thirty cycles of 1 min of denaturation (94°C), 1 min of annealing (56°C), and either 50 s (amplicons A and B), 1 min 50 s (amplicon C), or 3 min (amplicon D) of extension (72°C) were carried out. A final 7-min extension (72°C) step was added. All PCR products were electrophoresed in agarose gels containing 0.5× TBE buffer (1× TBE buffer contains 90 mM Tris, 90 mM boric acid, and 2 mM EDTA [pH 8.0]) and 0.5 μg of ethidium bromide per ml.

Cycle sequencing of amplicons A and B. Sequencing of the PCR products was done by the University Core DNA Services (Health Science Centre, University of Calgary). The process utilizes automated Applied Biosystems sequencing and a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Conditions were maintained as recommended, which included doubling of all reagents and use of 5% dimethyl sulfoxide in some, but not all of the reactions. Nested sequencing primers were used. The forward and reverse primers, respectively, were 5′tactcctgactgaccgatag3′ and 5′ggctagatcaccgggtttcg3′ for amplicon A and 5′cctgcgcggaagatgtaacg3′ and 5′gcattcgcacttctgatacc3′ for amplicon B. All sequencing templates and sequencing primers were gel purified.

RNA isolation, electrophoresis, Northern (RNA) blotting, and methylene blue staining. Cells were grown with shaking in Luria-Bertani broth and isolated at mid-log phase by centrifugation at 5,000 × *g* for 15 min at 4°C. Cells (2×10^9) were resuspended in 3 ml of extraction buffer (10 mM sodium acetate, 0.15 M sucrose, pH 4.8) at 4°C. Sodium dodecyl sulfate was added to a final concentration of 1%, and the solution was vortexed. A 3-ml volume of phenol at 65°C (sodium acetate buffered [pH 4.8]) was added, and the solution was vortexed, incubated at 65°C for 5 min, incubated at 0°C for 5 min, and centrifuged at 5,000 × *g* for 30 min at 4°C. The phenol extraction was repeated with the aqueous phase and followed by a final extraction with chloroform. The RNA was precipitated overnight in 2.5 volumes of ethanol at −20°C and centrifuged. The RNA pellet was washed with 75% ethanol and resuspended in DNase I buffer (50 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$) with 5 mM dithiothreitol. To this solution, 14 U of DNase I and 350 U of rRNasin were added, and the solution was incubated for 30 min at 37°C, extracted with an equal volume of chloroform, precipitated with 2.5 volumes of ethanol, and stored at −70°C.

RNA was electrophoresed through 1.2% agarose by using the glyoxal-dimethyl sulfoxide denaturation method (28) and blotted onto Hybond-N+ as recommended by the manufacturer (Amersham). The blots were stained for RNA by using 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2), washed several times in water, and allowed to air dry.

Computer methods. Each sequence was folded to minimize free energy by using the Genetics Computer Group version 8 implementation of FoldRNA (34) and the energies defined by Freier et al. (6). The results were visualized by LOOP VIEWER (7) on a Macintosh II/SI computer. GCG GAP, which minimizes gaps when aligning sequences, was used to generate identity comparisons.

RESULTS

IVSs in the seven *rrl* genes of *S. typhimurium* LT2. To elucidate the presence of IVSs, DNA fragments containing specific *rrl* genes isolated following PFGE were amplified for amplicons A (which contains helix-25) and B (which contains helix-45), and the products were separated by agarose gel electrophoresis (Fig. 2A). Amplicon A, obtained by using DNA containing *rrlA*, *rrlB*, *rrlC*, *rrlD*, or *rrlE* as the template, corresponded to the size of the amplicon resulting when genomic DNA of *E. coli* was used; this size is predicted to be 731 bp according to the known sequence of *rrlB* of *E. coli* (23). This result indicates that these *rrl* genes do not have a helix-25 IVS. Amplicons obtained by using template DNA containing *rrlG* or *rrlH* were ~110 bp larger (Fig. 2A); this suggests that these two *rrl* genes contain helix-25 IVSs. Amplicon B (containing helix-45), obtained by using template DNA containing *rrlA*, *rrlB*, *rrlC*, *rrlD*, *rrlE*, or *rrlH*, was larger than the amplicon resulting from amplification of *E. coli* DNA (calculated from the sequence to be 733 bp long [23]) by ~90 bp, indicating that each of these *rrl* genes contains a helix-45 IVS. Amplicon B made

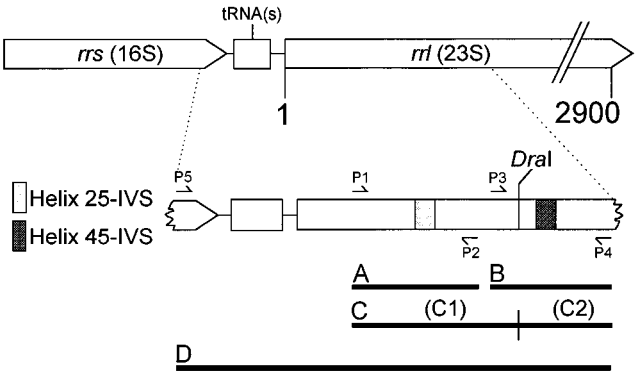


FIG. 1. One *S. typhimurium* LT2 *rrn* operon determines one 16S rRNA (*rrs*), one or two tRNAs, and one 23S rRNA (*rrl*). Considering several *E. coli rrn* operon sequence identities, four primer sets were constructed to PCR amplify amplicons A, B, C, and D. Amplicon A used primers P1 and P2; amplicon B used primers P3 and P4; amplicon C used primers P1 and P4; amplicon D used primers P5 and P4. Amplicons A (731 bp without an IVS, according to the *rrnB* gene sequence [23]) and B (733 bp without an IVS) were sequencing templates for nested primers in the helix-25 and helix-45 regions, respectively. Amplicon C was used to determine IVSs in the whole genome. Restricting C with *Dra*I yielded two fragments from each *rrl* gene: the C1 fragment (1,008 bp without an IVS) reported the presence of the helix-25 IVS (~110 bp), and the C2 fragment (551 bp without an IVS) reported the presence of the helix-45 IVS (~90 bp). Therefore, the genomic distribution of IVSs among the seven *rrl* genes was determined from the length and intensity variance of four fragments. Amplicon D (2,560 bp without IVSs) was used to retrieve *rrlH* amplicons A and B from a mixed template; the *rrl* gene of *rrnE* was copurified with *rrlH* on the 1,580-kb *Bln*I fragment. Amplification of D required the 3′ end of the *rrs* (16S) gene, which effectively eliminated *rrlE* (incomplete *rrn* operon) but included *rrlH* (complete *rrn* operon). *rrlH* amplicon D was gel purified and isolated in the same manner as the PFGE fragments and used as a template for amplicons A and B.

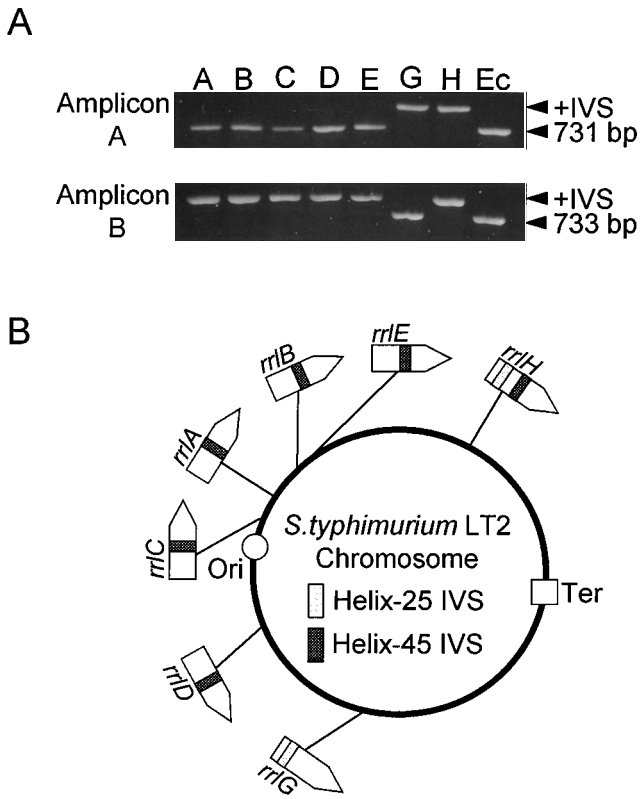


FIG. 2. (A) The DNA fragments resulting from amplification with the *rrl* genes (lanes A to H) of *S. typhimurium* LT2 as templates. Amplicon A contains helix-25, and B contains helix-45 (Fig. 1). Amplicons A and B each may be a shorter fragment (indicating no IVS in the template *rrl* gene) or a longer fragment (indicating an IVS). For lane Ec, *E. coli* K-12 genomic DNA was used as the template. The predicted size of *E. coli* amplicon A is 731 bp, and that of amplicon B is 733 bp; on the basis of the *E. coli rrlB* sequence (23). (B) Orientation and position of each *S. typhimurium* LT2 *rrl* gene on the chromosome (derived from reference 21) and the type(s) of IVS(s) detected in each *rrl* gene. The approximate locations of the origin of replication (Ori) and terminus (Ter) are indicated. Those positions in the *rrl* genes that do not contain IVSs contain small tetraloops, similar to *E. coli.*

with template DNA from *rrlG* resembles that from *E. coli*. These data indicate that *rrlG* has one IVS in helix-25, *rrlH* has IVSs in both helices, and the other five *rrl* genes have IVSs only in helix-45. The positions of the seven *rrl* genes on the genetic map of *S. typhimurium* LT2 (21) and the IVSs they carry on the basis of the data in Fig. 2A, are illustrated in Fig. 2B.

Nucleotide sequences and potential secondary structures of the helix-25 region. To confirm the presence or absence of IVSs in the amplicon A products, cycle sequencing of the seven helix-25 regions from both strands was completed. Each resulting sequence was folded into a potential RNA secondary structure to minimize free energy (Fig. 3A). All helix-25 regions form tight secondary structures where the first and last eight bases of each structure are conserved, including the *E. coli rrlB* tetraloop. The *S. typhimurium* LT2 helix-25 tetraloops from *rrlA*, *rrlB*, *rrlC*, *rrlD*, and *rrlE*, none of which contains IVSs as predicted by Fig. 2A, were identical, but a comparison with *E. coli* revealed that *S. typhimurium* contains a two-nucleotide addition and several base changes in and around the cap of the tetraloop. This is consistent with the finding that helix-25 cap nucleotide sequences are highly variable, to the point of being random, when compared in 42 eukaryotic, archeabacterial, and eubacterial-chloroplast sequences (5). *rrlG* and *rrlH* were confirmed to contain IVSs, of ~110 bp, inserted in helix-25. These IVSs show a low sequence identity of only 56% (Table 2), and their predicted secondary structures are very different (Fig. 3A). However, both IVSs maintain identity in the first and last 14 nucleotides, presumably because of conservation of the RNase III excision site, which was shown to occur in the first 16 nucleotides of the helix-45 IVS (4). The ability of RNase III to recognize a secondary structure (duplex stems) rather than a sequence consensus (27) accounts for the fact that the stems of helix-25 and helix-45 IVSs show limited sequence identity. *S. arizonae* helix-25 (4) is 56 and 94% identical to *rrlG* and *rrlH*, respectively (Table 2). A BLAST search (1) of nucleotide sequences in GenBank (release 84) against each IVS did not identify significant matches with any nucleotide sequences other than the IVSs in Tables 2 and 3.

Nucleotide sequences and potential secondary structures of the helix-45 region. All of the *rrl* genes of *S. typhimurium* LT2 except *rrlG* contained nucleotide sequences with helix-45 IVSs (Fig. 3B). The helix-45 tetraloop of *E. coli rrlB* and *S. typhimurium* LT2 *rrlG* are identical except for one nucleotide in the cap. As predicted from Fig. 2A, the nucleotide sequence shows that *rrlA*, *rrlB*, *rrlC*, *rrlD*, *rrlE*, and *rrlH* have IVSs of ~90 bp inserted in helix-45. These helix-45 IVSs are 100% identical in all six of these *rrl* genes, and they are also identical to those previously reported for *S. typhimurium* (4, 29). The *S. arizonae* helix-45 IVS (4) is 85% identical to *S. typhimurium*, and *Y. enterocolitica* helix-45 IVSs from strains of groups 1 and 2 (29) are 61 and 87% identical, respectively, to helix-45 IVSs of *S. typhimurium* (Table 3).

IVSs in the *rrl* genes of 21 wild-type strains of *S. typhimurium*. Genomic DNA, which contains all seven *rrl* genes, was isolated from 21 independent wild-type strains of *S. typhimurium* from the SARA set. Amplicon C (Fig. 1), produced with this genomic DNA, was *Dra*I restricted and separated by agarose gel electrophoresis (Fig. 4A). Fragment length and intensity variance revealed the approximate number and type of IVSs present throughout the seven *rrl* genes. The C1 fragments from *Dra*I digestion contained helix-25 (Fig. 1); the shorter fragment, estimated from the *E. coli rrnB* nucleotide sequence (23) to be 1,008 bp long, indicates amplification from an *rrl* gene lacking an IVS, and the longer fragment indicates amplification from an *rrl* gene possessing an IVS. The high intensity of the shorter fragment and the low intensity of the longer fragment obtained with strain LT2 (=SARA2), which has five *rrl* genes lacking the helix-25 IVS and two possessing the IVS (Fig. 2), were as expected. The C1 fragment patterns of strains of groups 1 and 3 were similar to that of strain LT2, suggesting that both have two helix-25 IVSs; however, group 2 was less intense than group 1 or 3, and this indicates that it possesses only one helix-25 IVS. Strain SARA21 of group 4 does not carry helix-25 IVSs, as it produced only one C1 fragment, equivalent in size to that obtained from *E. coli* genomic DNA.

The C2 fragments from *Dra*I digestion contained helix-45 (Fig. 1). LT2 DNA showed a low-intensity fragment with a size equivalent to the 551-bp fragment expected for amplification of *E. coli* DNA (23) and a high intensity fragment, ~90 bp larger, indicating amplification from an *rrl* gene possessing an IVS. As expected, the intensity of the larger fragment was about six times as great, since six of seven *rrl* genes have the helix-45 IVS (Fig. 2). All of the strains in groups 1 and 4 showed similar C2 fragment patterns, indicating that all have six *rrl* genes with IVSs. Strains in group 2 contained fewer helix-45 IVSs than did those in groups 1 and 4, and strains in group 3 have helix-45 IVSs in all of their *rrl* genes.

RNA isolated from each SARA group representative sup-

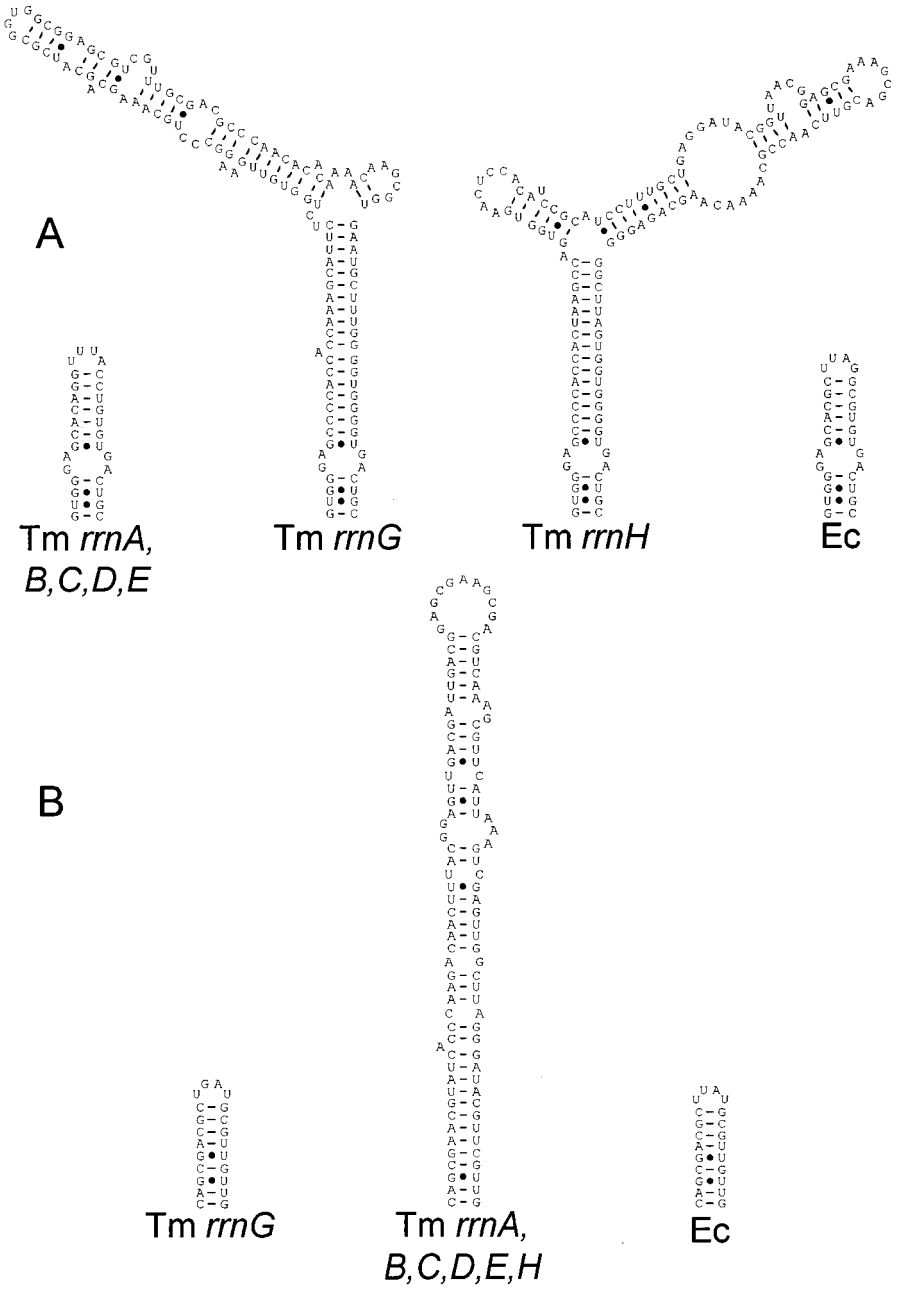


FIG. 3. Potential RNA secondary structures of all helix-25 and helix-45 nucleotide sequences from *S. typhimurium* LT2 (Tm). *rrl* genes that are grouped together are 100% identical. The *E. coli* (Ec) sequences for helix-25 and helix-45 of *rrlB* (23) are shown. (A) Helix-25 regions. (B) Helix-45 regions. The free energy calculated at 37°C for the *rrlG* helix-25 IVS was −54.5 kcal/mol, that for the *rrlH* helix-25 IVS was −52.2 kcal/mol, and that for the helix-45 IVS was −37.9 kcal/mol. Filled circles represent guanosine-uridine base pairings. The GenBank accession numbers are U43699 (helix-25 *rrlG* IVS), U43670 (helix-25 *rrlH* IVS), and U49921, U49922, U49923, U49924, U49925, and U49926 for helix-45 *rrlA*, *rrlB*, *rrlC*, *rrlD*, *rrlE*, and *rrlH* IVSs, respectively.

ports and extends the conclusions drawn from PCR analysis (Fig. 5A). Group 1 contains the characteristic LT2 23S rRNA fragmentation pattern, producing 2.4-, 1.7-, 1.2-, 0.7-, and 0.5-kb rRNA fragments but no intact 23S (2.9-kb) rRNA (Fig. 5C contains a fragmentation schematic). In group 2, which, according to PCR data (Fig. 4A), has only one *rrl* gene with a helix-25 IVS and fewer helix-45 IVSs than group 1, rRNA of 2.9 kb was detected, rRNA of 0.7 kb was missing, and rRNA of 0.5 kb was reduced (Fig. 5A); this extends the PCR data, indicating that one of the *rrl* genes has a helix-25 IVS only, some (probably four) have helix-45 IVSs only, and some (prob-

TABLE 2. Comparison of *S. typhimurium* LT2 helix-25 IVS with other helix-25 IVSs

Helix-25 IVS source	% Nucleotide sequence identity[a] with helix-25 IVS of:		
	S. arizonae	*S. typhimurium* LT2 *rrlG*	*S. typhimurium* LT2 *rrlH*
S. arizonae			
S. typhimurium LT2 *rrlG*	56		
S. typhimurium LT2 *rrlH*	94	56	

[a] GCG GAP was used to align sequences for identity comparisons.

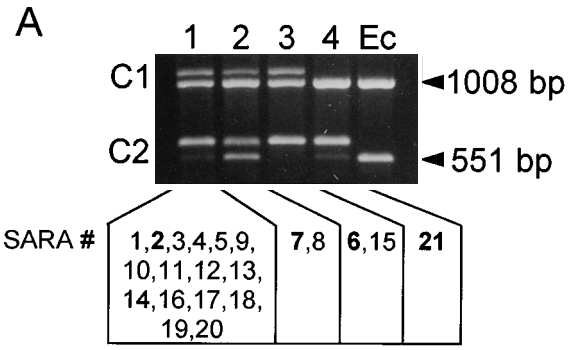
TABLE 3. Comparison of *S. typhimurium* LT2 helix-45 IVS with other helix-45 IVSs

Helix-45 IVS source	% Nucleotide sequence identity[a] with helix-45 IVS of:			
	S. arizonae	*S. typhimurium* LT2	*Y. enterocolitica* group 1	*Y. enterocolitica* group 2
S. arizonae				
S. typhimurium LT2	85			
Y. enterocolitica group 1	66	61		
Y. enterocolitica group 2	83	87	59	

[a] GCG GAP was used to align sequences for identity comparisons.

ably two) have no IVSs. Group 3 lacks the 2.4-kb rRNA fragment and contains more 0.7- and 0.5-kb rRNA fragments than does LT2 (Fig. 5A). This verifies that group 3 contains helix-45 IVSs in all *rrl* genes (thus, it does not produce a 2.4-kb rRNA fragment) and has two *rrl* genes containing both helix-25 and helix-45 IVSs, as predicted from PCR data (Fig. 4A). PCR data also show that group 4 contains no helix-25 IVSs but several helix-45 IVSs; as expected, some intact 23S, 1.7-kb, and 1.2-kb rRNAs were observed, but there were no 2.4-, 0.7-, or 0.5-kb rRNA fragments, indicating that six genes have helix-45 IVSs and one has no IVS (Fig. 5A).

Amplification of genomic DNAs from seven different LT2 lines, followed by *Dra*I digestion, yielded indistinguishable fragment sizes and intensities for all lines except LB5010, which contains less C1 fragment with IVSs than does LT2, indicating that it has only one helix-25 IVS, not two (Fig. 4B). rRNA from LB5010 has no 0.7-kb fragment, while the 1.2- and 0.5-kb fragments are increased and reduced, respectively; this indicates



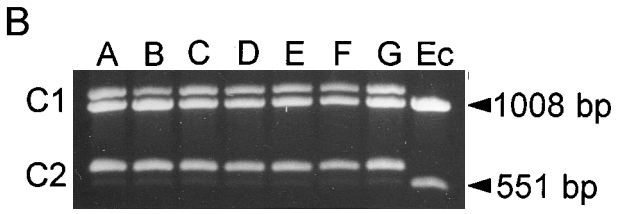
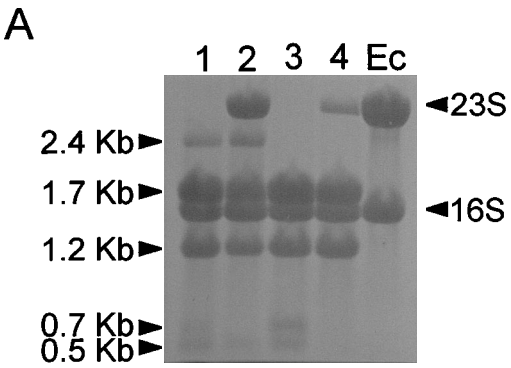
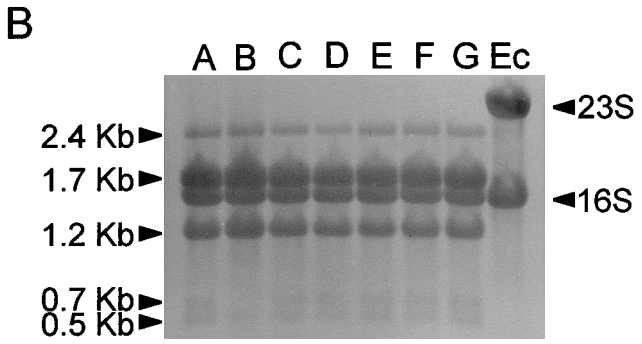


FIG. 4. *Dra*I restriction of amplicon C, which was obtained by using whole genomic DNA as the template. PCR products were GlassMAX purified and quantified by spectrophotometer, and then 600 ng was digested with *Dra*I. (A) Amplification of genomic DNAs of 21 wild-type *S. typhimurium* strains from SARA; these can be separated into four visually distinct groups (lanes 1 to 4). The SARA set numbers in boldface were used as representatives of the groups. SARA2 represents strain LT2. The restriction patterns were standardized by using *E. coli* (lane Ec) genomic DNA as the template: the C1 fragment (calculated to be 1,008 bp long according to the *rrnB* gene sequence [23]) and the C2 fragment (551 bp). (B) Amplification of genomic DNAs of seven different lines of *S. typhimurium* LT2 (lanes A to G) and *E. coli* K-12 (lane Ec). Lane A contained SGSC1412, the strain used as the wild type of LT2 at the SGSC; it was stocked in 1955 as a lyophil, obtained by the SGSC in 1985 from J. Lederberg, and stored since then as a lyophil at −70°C. Lane B contained strain LB5010 (multiply auxotrophic, host restriction deficient), obtained from L. Bullas (3). Lane C contained wild-type TN3618, obtained from L. Miller. Lane D contained wild-type SA4254, obtained from C. Higgins. Lane E contained wild-type SL937, obtained from B. Stocker. Lane F contained wild-type LT2A, obtained from B. Ames. Lane G contained wild-type EB360, obtained from E. Barrett.





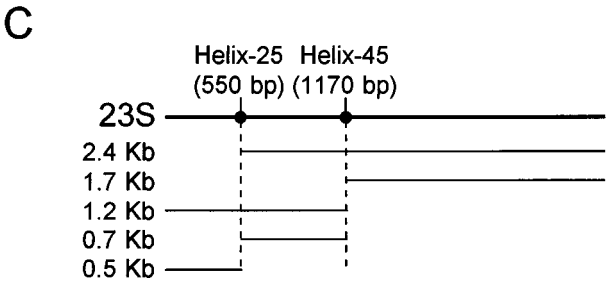


FIG. 5. rRNAs of strains of *S. typhimurium*. A 10-μg sample of RNA was separated by 1.2% agarose glyoxal–dimethyl sulfoxide denaturing gel electrophoresis, blotted onto Hybond-N+, and stained with methylene blue. (A) rRNAs from groups 1 (SARA2), 2 (SARA7), 3 (SARA6), and 4 (SARA21) of the SARA set, as defined in the legend to Fig. 4A. Group 1 contains the characteristic LT2 23S rRNA fragmentation pattern, producing 2.4-, 1.7-, 1.2-, 0.7-, and 0.5-kb rRNA fragments. The positions of the 23S and 16S rRNAs of *E. coli* K-12 (lane Ec) are indicated. (B) rRNAs isolated from seven different lines of *S. typhimurium* LT2 (lanes A to G) and *E. coli* K-12 (lane Ec). Lanes: A, SGSC1412; B, LB5010; C, TN3618; D, SA4254; E, SL937; F, LT2A; G, EB360. (C) 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 bp 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 bp 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.

that the helix-25 IVS in the *rrl* gene has been lost. The remaining six LT2 lines are identical with respect to rRNA fragmentation. Although these seven lines all originated from the same LT2 wild type, they have been maintained in different laboratories since the 1950s and separated by numerous single-colony isolations over 30 years. This shows that IVSs in specific strains are fairly stable.

DISCUSSION

How are IVSs distributed to different cells, and how are they propagated to more than one of the seven *Salmonella rrl* genes? Each IVS might evolve independently because of strong selective pressures, but this seems unlikely since there is no evidence of a functional role. IVSs are distributed sporadically among many bacterial genera and species, indicating that they have been distributed by "lateral transfer" from genetic exchange. The two helix-25 IVSs in *rrlG* and *rrlH* of *S. typhimurium* LT2 are only 56% identical, but the latter is 94% identical to the helix-25 IVS from *S. arizonae* (Table 2). The IVSs in helix-45 of *S. typhimurium*, *S. arizonae*, and *Y. enterocolitica* group 2 all show >80% nucleotide identity, while *Y. enterocolitica* group 1 is less related to all of the others (Table 3). These data suggest that the two helix-25 IVSs and one helix-45 IVS entered *S. typhimurium* LT2 in three independent lateral-transfer events.

Propagation of IVSs to different *rrl* genes within the same cell might occur by either of two methods. One is double-reciprocal recombination, normally *recA* dependent, between two genes present on separate sister chromatids immediately after replication. The *rrn* genes are good targets for such an exchange, since they include at least 500 bp of homologous DNA on each side of the IVSs. This method can exchange IVSs between different *rrl* genes, but the reciprocal exchange cannot increase the number of IVSs unless it is between an IVS *rrl* gene and a non-IVS *rrl* gene on separate chromatids.

The second method is gene conversion, i.e., nonreciprocal transfer of information from one DNA duplex to another, which may occur by a double-strand break-and-repair system after replication (31). Gene conversion occurs commonly in eukaryotes, such as yeasts to maintain homogeneity within a family of repeated sequences and, on rare occasions, allows a variant sequence to replace an existing sequence; it therefore has the potential for directed change and facilitates correction without changing the gene dosage (22). Conversion is less common in bacteria, where the DNA is mostly unique, but it occurs between the *rrn* operons, which are members of a multigene family (9, 10). In addition, conversion of the *Salmonella* phase 1 flagellin gene *fliC* to the phase 2 gene *fliB* in *E. coli* has been demonstrated (24), and the *catIJF* and *pcaIJF* genes of *Acinetobacter calcoaceticus*, which have nearly identical nucleotide sequences, convert each other in a *recA*-dependent process at rates of about 10^{-6} (8, 16). The following points support the hypothesis that dispersion and sequence maintenance of IVSs is due to gene conversion. (i) IVSs in six of the seven *rrl* genes of *S. typhimurium* LT2 have identical sequences (Fig. 3). These IVSs do not originate by replication from parent cells, since IVSs occur sporadically, but must have entered a single *rrl* gene by lateral transfer and been propagated by gene conversion. (ii) We expected that sequences of IVSs diverge by random mutation, yet the sequences of all helix-45 IVSs are identical in strain LT2, although they differ from IVSs in *S. arizonae* and *Y. enterocolitica* (Table 2). This indicates that IVSs are maintained by gene conversion, as in eukaryotes (22); this could also explain why all helix-25 tetraloops in strain LT2 are identical.

IVSs are relatively stable, for most SARA set members and six of seven separate lines of *S. typhimurium* LT2 possess the same number of IVSs in both helix-25 and helix-45 (Fig. 4A and B). The LT2 lines have been separated from each other by many single-colony isolations over a period of more than 30 years. However, IVS possession has changed significantly in some wild-type strains of *S. typhimurium* (Fig. 4 and 5).

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