

Role of Genomic Rearrangements in Producing New Ribotypes of *Salmonella typhi*

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***Salmonella typhi* is the only species of *Salmonella* which grows exclusively in humans, in whom it causes enteric typhoid fever. Strains of *S. typhi* show very little variation in electrophoretic types, restriction fragment length polymorphisms, cell envelope proteins, and intervening sequences, but the same strains are very heterogeneous for ribotypes which are detected with the restriction endonuclease *Pst*I. In addition, the genome of *S. typhi* has been proven to undergo genomic rearrangement due to homologous recombination between the seven copies of *rrn* genes. The relationship between ribotype heterogeneity and genomic rearrangement was investigated. Strains of *S. typhi* which belong to 23 different genome types were analyzed by ribotyping. A limited number of ribotypes were found within the same genome type group; e.g., most strains of genome type 3 belonged to only two different ribotypes, which result from recombination between *rrnH* and *rrnG* operons. Different genome type groups normally have different ribotypes. The size and identity of the *Pst*I fragment containing each of the seven different *rrn* operons from *S. typhi* Ty2 were determined, and from these data, one can infer how genomic rearrangement forms new ribotypes. It is postulated that genomic rearrangement, rather than mutation, is largely responsible for producing the ribotype heterogeneity in *S. typhi*.**

Salmonella typhi grows only in humans, in whom it causes typhoid enteric fever. Independent *S. typhi* strains from different geographic regions are phenotypically homogeneous. Reeves et al. (21) showed that 26 strains of *S. typhi* tested by multilocus enzyme electrophoresis had an identical electrophoretic type, leading to the conclusion that *S. typhi* strains are a single clone. Selander et al. (25) also found *S. typhi* more homogeneous in electrophoretic type than other species of *Salmonella*, although they identified two electrophoretic types, Tp1 and Tp2. Data on restriction fragment length polymorphisms from digestion with *Eco*RI and *Pst*I showed conserved banding patterns for all 22 *S. typhi* strains studied (5). The cell envelope protein profiles for a series of outer membrane and inner membrane proteins for 32 *S. typhi* strains showed only very minor differences (6). Each of 15 *S. typhi* strains had intervening sequences in all seven *rrl* genes for rRNA, and all those tested had identical sequences (18). All these data show a high degree of homogeneity of *S. typhi* strains.

Although the above data indicate homogeneity, ribotyping studies of *S. typhi* by Altwegg et al. (1), Nastasi et al. (19), and Pang et al. (20) found a large number of ribotypes (RTs) among different strains of *S. typhi*, whereas other *Salmonella* spp. are relatively homogeneous in RTs. Bacteriophage typing with Vi phage is the most common method used to demonstrate epidemiological associations of *S. typhi* strains (2), but RT data have also been very valuable for further subdivision of the different phage types (1, 19, 20). The objective of this study was to determine the basis for heterogeneity of RTs in *S. typhi*.

RTs are determined by probing a Southern blot of a restriction digest of the genome with ribosome sequences; thus, the RT of a strain is a specific pattern of band sizes, each band containing rRNA sequences. In enteric bacteria such as *Sal-*

monella (11) and *Escherichia coli* (4), which have seven *rrn* operons, seven fragments containing *rrn* operons are expected if digestion is performed with an enzyme such as *Pst*I, which does not digest within the *rrn* operon. Each fragment is composed of two arms (the distance from the left end, or 16S end, of the *rrn* operon to the nearest *Pst*I site, and the distance from the right end, or 5S end, of the *rrn* operon to the nearest *Pst*I site) plus the *rrn* operon itself, which is 6 kb; thus, all seven bands in *Pst*I digests that hybridize to the probe are 6 kb or larger. An RT is defined as a specific set of lengths of the seven fragments containing the seven *rrn* operons. (RTs for enzymes which cut within the *rrn* operon should have 14 fragments representing the two arms from each operon plus internal *rrn* fragments if any.) Changes in the fragment lengths with resultant changes in RT can result from (i) point mutations in the genome, leading to gain or loss of restriction sites in one of the two arms of the restriction fragment carrying the *rrn* operon (nucleotide sequences within the *rrn* operon are highly conserved), or (ii) chromosomal rearrangements which affect the genome within the fragments carrying the *rrn* operons.

The structure and the order of genes on the chromosomes of different enteric bacteria are usually strongly conserved (9, 23); the genetic and physical maps of *S. typhimurium* LT2 (11), *E. coli* K-12 (4), *S. enteritidis*, and *S. paratyphi* B (10) are very similar. All fragments from digestion by the endonuclease I-*Ceu*I (which cuts only in *rrn* operons [13, 17]) are in the order ABCDEFG, as illustrated for genome type 1 (GT1) in Fig. 1A. Within each species, the genomic order of these fragments is also conserved, as exemplified by strains of *S. typhimurium* (13). However, the genome of *S. typhi* is frequently rearranged by recombination between *rrn* operons; by using partial digestion by I-*Ceu*I, 21 different orders were detected among 127 wild-type strains examined (14, 16). These different orders of I-*Ceu*I fragments are defined as GTs and are shown in detail in reference 16. They are illustrated by GT9 (Fig. 1B) and GT3 (Fig. 1C and D), in which the order of fragments is changed. We postulated that homologous recombination between *rrn* operons results in translocations and inversions; for example, in *S. typhi* Ty2, which is GT9 (Fig. 1B), linkages of genes are

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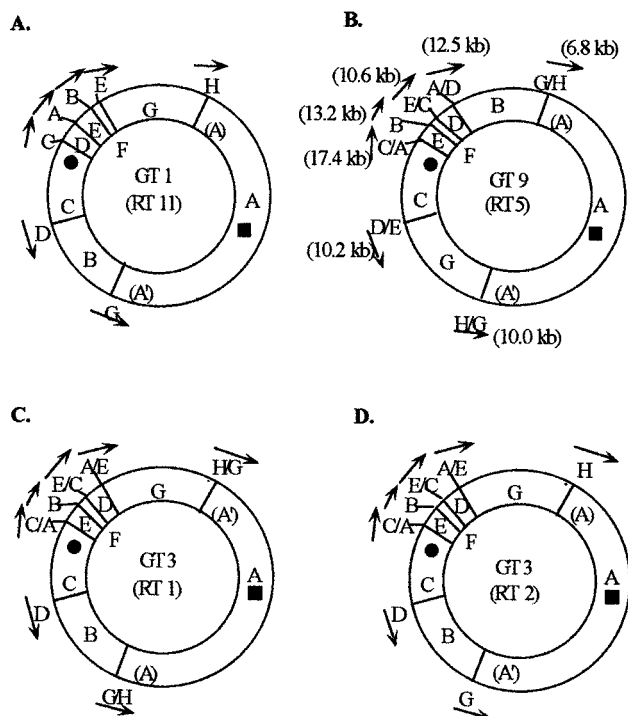


FIG. 1. Genomic rearrangements of the *I-CeuI* fragments of GT1, GT3, and GT9 in *S. typhi* (12, 14). The RTs of each GT are also shown. The letters for each *I-CeuI* fragment are within or adjacent to the circles, and each junction between the fragments is the endonuclease cleavage site of *I-CeuI* (11). The letters outside the circle indicates the *rm* genes (in GT1) and inferred *rm* recombinants (in GT3 and GT9). The solid circle in *I-CeuI*-C denotes the origin of replication (*oriC*), and the square in *I-CeuI*-A denotes the termination of replication (TER). Arrows beside the *rm* operons indicate the orientation from *ms* (for 16S rRNA) to *rrf* (for 5S rRNA). The order of these *I-CeuI* fragments on the chromosome of strains of *S. typhi* was determined by the partial-digestion method from data reported previously (12, 14, 16). (A) GT1 is the same as the order in *S. typhimurium* LT2 and *E. coli* K-12. The positions of the *rm* operons are shown. (B) GT9 is the GT found in Ty2, which has been commonly used as a wild-type strain (12). (C and D) GT3 is the most common GT found among *S. typhi* strains; it has two dominant ribotypes, RT1 and RT2.

changed so that fragment *I-CeuI*-B is now linked to the A end of *I-CeuI*-A while fragment G is linked to the A' end (a reversal of the order in GT1 [Fig. 1A]); this is postulated to be due to recombination between *rmH* and *rmG* in GT1, resulting in *rmG/H* and *rmH/G* in strain GT9 (Ty2) (12). Homologous recombination between *rmn* operons had previously been shown to occur in *S. typhimurium* LT2 (3) and *E. coli* K-12 (7) at frequencies as high as 10^{-4} per cell and to result in the formation of rearrangements of the types we have observed in *S. typhi*.

Homologous recombination between two *rm* operons is expected to change the RT determined from a *PstI* digest; the two "arms" of each of the two *PstI* fragments will be interchanged, resulting in two new fragment lengths, but the other five *PstI* fragments should remain unaltered. In this study, we tested the hypothesis that recombination between *rm* operons, rather than point mutation in *PstI* restriction sites, is responsible for the formation of new ribotypes in *S. typhi*. We found that recombination is the major basis for new RT formation, although some role for mutation cannot be excluded. The sizes of the *PstI* fragments of each of the seven *rm* operons of *S. typhi* Ty2 are identified, and recombination between *rmH* and *rmG* is shown to be common.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. A total of 127 *S. typhi* strains were obtained from different sources: Laboratory Centre for Disease Control, Ottawa, Canada; Centers for Disease Control and Prevention, Atlanta, Ga.; Provincial Laboratory of Alberta, Calgary, Canada; Tikki Pang (University of Malaya, Kuala Lumpur, Malaysia); Robert Selander (Pennsylvania State University); and Bruce Stocker (Stanford University). They were identified as *S. typhi* based on biochemical and antigenic characterizations, which were determined by the laboratories of origin and confirmed by the Laboratory Centre for Disease Control, Ottawa, Canada. Genomic analysis of these strains has been previously reported (16). The strains were grown on Luria-Bertani medium (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 3.5 ml of 1 M NaOH); solid medium also contained 1.5% agar. The minimal medium used is a modified Davis medium (24). Tetracycline was used at 20 μ g/ml. Strains were maintained in 15% glycerol at -70°C , and a single colony was isolated prior to use.

Enzymes and chemicals. Endonucleases were from New England Biolabs (*PstI*, *AvrII* = *BlnI*, *I-CeuI*, *SpeI*), and Boehringer Mannheim (*XbaI*). Other chemicals, such as agarose, were from GIBCO BRL.

Preparation, digestion, and separation of genomic DNA and Southern blotting. Preparation of high-molecular-weight genomic DNA, endonuclease cleavage of DNA embedded in agarose blocks, and separation by pulsed-field gel electrophoresis were as reported previously (10, 12).

PstI-restricted chromosomal DNA fragments of different strains of *S. typhi* were separated by conventional gel electrophoresis in Tris-borate-EDTA buffer for 20 h at 100 V. After electrophoresis, the gel was washed in 0.25 N HCl, then in 0.5 N NaOH-1.5 M NaCl, and finally in 1 M ammonium acetate-0.02 N NaOH. Separated DNA fragments in the gel were then transferred to a positively charged nylon membrane (Boehringer Mannheim) by Southern blotting.

The Southern blotting was done as follows. The gel and the positively charged nylon membrane were sandwiched between filter papers soaked in a buffer solution of 1 M ammonium acetate-0.02 N NaOH and blotted for 18 to 24 h. Then the membrane was dried at 80°C for 2 h to fix the DNA onto the membrane.

Preparation of the probe for ribotyping. The membrane was probed with plasmid pT711 containing the *E. coli rmbB* operon with the 16S and 23S rRNA and 5S tRNA gene sequences (25); this plasmid was present in strain SGSC2266, an *E. coli* K-12 strain. SGSC2266 was grown overnight on a plate containing Luria-Bertani agar plus 100 μ g of ampicillin per ml. Then the cells were scraped up and the plasmid DNA was isolated by the alkali lysis method as described by Sambrook et al. (22).

Digoxigenin DNA labeling and detection of RTs. The digoxigenin DNA labeling and detection methods were performed as described by the manufacturer (Boehringer Mannheim). The RTs were detected by exposing a film to the membrane.

Identification of the *rm* operon(s) in specific RT bands. The *XbaI*-*I-CeuI*-*BlnI*-*SpeI* genome map of *S. typhi* Ty2 has been developed previously (12). The first three endonucleases cut within the *rm* operons of Ty2, while *SpeI* cuts outside the *rm* operons; therefore, some restriction fragments contain a full copy of a *rm* operon, while others contain only part of an *rm* operon. The genome of Ty2 was digested with *XbaI*, *I-CeuI*, *BlnI*, or *SpeI*, and the restricted fragments were then separated by pulsed-field gel electrophoresis (12). Restricted fragments which include full or partial *rm* operons were then isolated by being excised from the gel. These fragments were then redigested with *PstI* and probed for *rm* operons.

RESULTS

RTs of *S. typhi* strains. All 127 strains of *S. typhi* reported previously (16) and briefly described in Materials and Methods were tested for RT following digestion by *PstI*; they were separated into 31 different RTs (Table 1), according to data of the type reported in Fig. 2A.

If changes in RTs are due to genomic rearrangements only and not to mutations, strains with the same genomic arrangement should have the same RT. We therefore examined genomic DNA of the 57 strains of *S. typhi* which are of GT3; a sample of 19 of these is shown in Fig. 2A, lanes 1 to 19. These 19 strains fell into three classes for RT; most strains were RT1 or RT2, while 1 was RT3. The cartoon in Fig. 2B shows the seven fragments of a representative strain of RT1 from lane 9, with predicted sizes in kilobases; a strain of RT2 from lane 4 and a strain of RT3 from lane 14 are also illustrated. The RT of strain Ty2 (GT9) is also shown for comparison. Among the whole set of 57 strains of GT3, 27 were of RT1, 18 were of RT2, 1 was of RT3, and 1 was of RT4 (only some of the data are shown). These data indicate that within a specific GT there

TABLE 1. RTs detected following *Pst*I digestion among the 127 strains of *S. typhi* belonging to different GTs

GT	Total no. of strains in GT	RT	No. of strains with specific RT
1	2	11	2
2	22	6	16
		7	2
		8	1
		9	2
		10	1
3	57	1	37
		2	18
		3	1
		4	1
4	5	2	1
		3	2
		12	1
		13	1
5	2	14	2
6	8	9	1
		15	7
7	3	13	2
		16	1
8	2	6	2
9	5	5	5
11	2	17	2
13	2	18	1
		19	1
14	2	20	1
		21	1
16	1	22	1
17	1	23	1
18	2	24	1
		25	1
19	4	23	4
22	2	26	2
23	2	16	1
		27	1
24	2	28	1
		29	1
25	1	30	1
26	1	31	1

is a limited number of different RTs; the sections below explain the basis for the occurrence of the different RTs.

The RTs of eight strains representing five different GTs are shown in Fig. 3. All these strains have different RTs, except for the two strains of GT11, both of which are RT17. The RTs of all 127 strains, which belong to 21 different GTs, are summa-

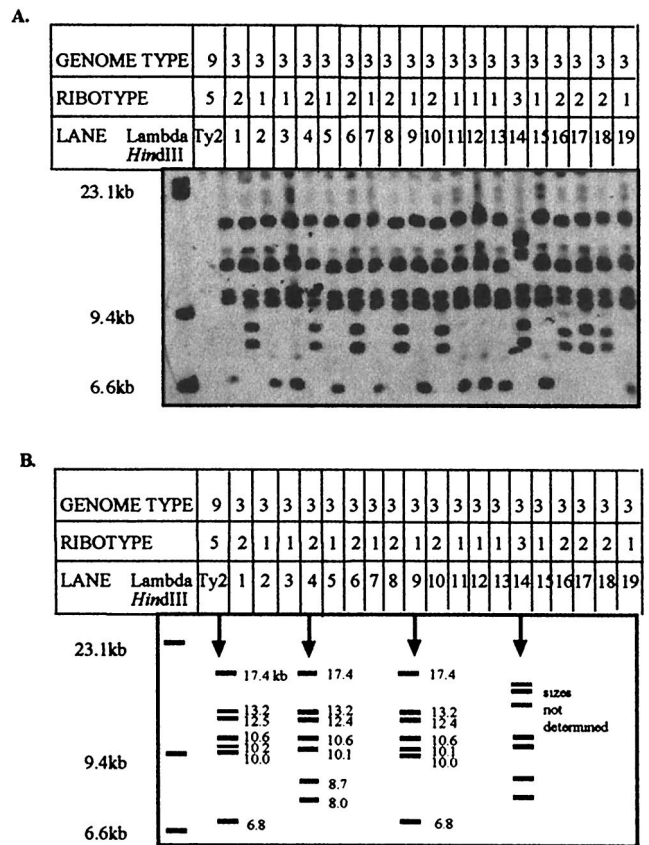


FIG. 2. (A) Chemiluminescent detection of RTs in some of the *S. typhi* strains in GT3. The DNA of the strains was first digested by the endonuclease *Pst*I, which cuts outside the *rm* genes, and then separated by conventional gel electrophoresis, Southern blotted, and probed. The standard sizes of lambda *Hind*III and the RT of Ty2 (RT5) are shown for comparison. Lanes 1 to 19 contain strains from GT3. The corresponding RTs are shown for all strains. Seven bands (some appears as doublets) are detected in each RT. Within GT3, the strains show two dominant ribotypes (RT1 and RT2), and one strain shows RT3. Genomic DNA of the following strains are in the indicated lanes: 1, 26T4; 2, 26T7; 3, 26T11; 4, 26T15; 5, 26T18; 6, 26T22; 7, 26T23; 8, 26T29; 9, 26T30; 10, 26T33; 11, 26T34; 12, 26T35; 13, 26T36; 14, 26T41; 15, 26T42; 16, 26T43; 17, 26T45; 18, 26T47; 19, 25T37. (B) Proposed fragment sizes observed in panel A. The sizes of lambda *Hind*III are 23.1, 9.4, and 6.6 kb. The fragment sizes for strain Ty2 (RT5, GT9), for a single strain of RT2 and GT3 (lane 4) and a single strain of RT1 and GT3 (lane 9) are shown, as well as the fragments of the one strain of RT3 of GT3 (lane 14).

riized in Table 1. These data show that strains with different GTs almost always have different RTs.

Identification of the specific *rm* operon associated with each fragment in the RT. If the formation of new RTs is due to homologous recombination between different *rm* operons alone, strains which are defined as having the same GT by the method of partial *I-Ceu*I digestion used by Liu and Sanderson (16) should all have similar RTs. However, they may not be identical, because the partial *I-Ceu*I analysis method cannot detect inversions between *rmC* and *rmD* on either end of fragment *I-Ceu*I-C or between *rmG* and *rmH* on either end of *I-Ceu*I-A (Fig. 1A) (see also references 14 and 16). Thus, inversions of these types might occur within GT3. We therefore determined the specific *rm* operon associated with each RT fragment in *S. typhi* Ty2, to see if the fragments which vary within a GT are the ones which we would predict. This was done by performing pulsed-field gel electrophoresis and then excising agarose blocks containing DNA which contains indi-

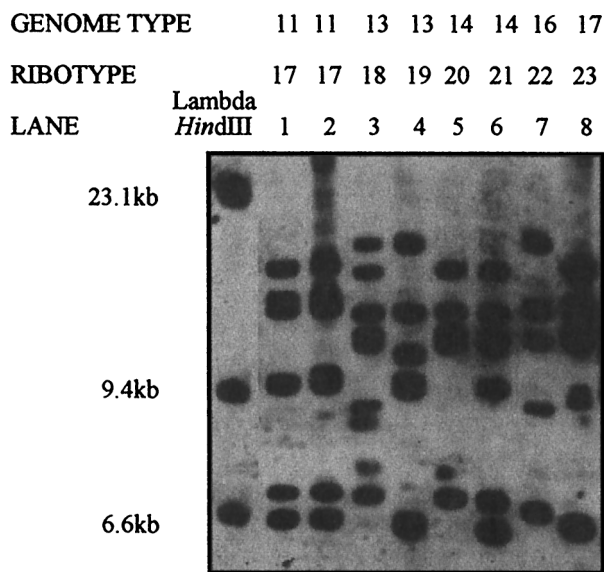


FIG. 3. Chemiluminescent detection of RTs in *S. typhi* strains which belong to different GTs. The genomic DNA of these strains was treated as described in the legend to Fig. 2. The standard sizes of lambda *Hind*III are shown. Lanes: 1 and 2, 26T49 and T189 (both GT11); 3 and 4, 382-82 and SA4865 (both GT13); 5 and 6, 26T38 and R16B7 (both GT14); 7, 26T9 (GT16); 8, In4 (GT17). The corresponding RTs are shown for all strains.

vidual fragments that carry known *rrn* operons of *S. typhi* Ty2, as described in Materials and Methods; these fragments were then digested with *Pst*I, electrophoresed, and probed. Representative data are given in Fig. 4A, and interpretations are provided in Fig. 4B. For example, lane 1 contains DNA of fragment *Spe*I F, known from earlier studies (12) to carry *rrnG/H*; this yields a single band of 6.8 kb, and so the 6.8-kb band carries *rrnG/H*. Lane 3 contains *Spe*I-AA, known to carry *rrnH/G*; this yields a single band of 10.0 kb, and so the 10.0-kb band carries *rrnH/G*. These two fragments from strain Ty2, of 6.8 and 10 kb, are also present in strains of RT1 in GT3; however, they are missing from RT2, where they are replaced by fragments of 8.0 and 8.7 kb, while all other fragments remain unaltered between RT1 and RT2. Our conclusion is that strains of RT1 carry the inversion of fragment I-*Ceu*I-A which is present in Ty2, while strains of RT2 have the "normal" orientation of this fragment, which is present in *S. typhimurium* and most other enteric bacteria; these two orientations are illustrated in Fig. 1C and D. Thus the only difference in RT fragments between RT1 (37 strains) and RT2 (18 strains) can be explained by an inversion between *rrnG* and *rrnH*; no mutations in *Pst*I sites needs to be invoked to explain the RTs of all these strains. RT3 (Fig. 2, lane 14) and RT4, however, cannot be thus explained, and mutation might be invoked to explain some of the fragments observed.

Fragments carrying each of the seven *rrn* operons of *S. typhi* Ty2, either alone or (in some cases) in combination, were isolated, digested with *Pst*I, and probed (Table 2). In some cases, either the left or right arm of the *Pst*I fragment and part of the *rrn* operon were included because the *rrn* operon was digested by the enzyme used. The sizes of the *Pst*I fragment including each *rrn* operon, derived from these data, are illustrated in Fig. 1B for strain Ty2.

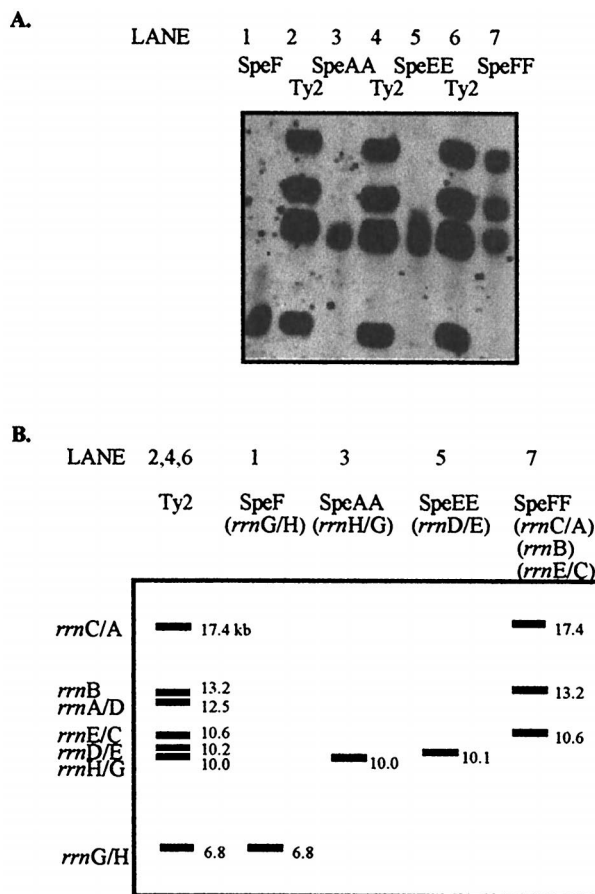


FIG. 4. (A) Chemiluminescent detection of *rrn* operons from *S. typhi* Ty2 following isolation of individual genomic fragments from pulsed-field gel electrophoresis, digestion by *Pst*I, separation by conventional gel electrophoresis, Southern blotting, and probing. Lanes: 2, 4, and 6, whole genomic DNA of Ty2; 1, *rrnGH* from the *Spe*I-F fragment (see Fig. 1 in reference 12); 3, *rrnHG* from *Spe*I-AA; 5, *rrnDE* from *Spe*I-EE; 7, *rrnCA*, *rrnB*, and *rrnEC* from *Spe*I-FF. The identity of the individual *rrn* operon can be determined from the genomic cleavage map of Ty2 as reported previously (12). (B) Interpretation of the above results; the sizes of the bands are shown.

DISCUSSION

Homologous recombination between the *rrn* operons results in rearrangements of the DNA fragments between these *rrn* operons, causing the formation of duplications, deletions, transpositions, and inversions, as illustrated in Fig. 4 of reference (16). These rearrangements can also produce new RTs, since they bring together different *Pst*I fragment lengths. Such RTs, in principle, can also result from mutations in the *Pst*I target sites. However, we conclude that the diversity of RTs results primarily from genomic rearrangements rather than from mutations in the *Pst*I sites, based on the following data.

(i) Among the 57 strains which belong to GT3, 37 were RT1, 18 were RT2, and only 1 was RT3 and 1 was RT4. We concluded that RT1 and RT2 differ only in fragments which we have identified to be due to the postulated recombination between *rrnH* and *rrnG*. Recombination between these *rrn* operons in wild-type strains has been observed before; they are recombined in *S. typhi* Ty2 (12) and in *S. paratyphi* A (15). Recombination can occur only between *rrn* operons with the same polarity; thus, inversions cannot occur within the *rrn* operons in the half of the chromosome in which the *rrn* operons are oriented in the same direction, but they can occur

TABLE 2. The restriction fragments containing specific *rm* operons isolated from *S. typhi* Ty2 following digestion with *SpeI*, *XbaI*, and *BlnI*^a

<i>rm</i> operon	I-CeuI junction fragment	Restriction fragment	Fragment size (kb)
<i>rmGH</i>	BA	<i>BlnI</i> -C	6.8
<i>rmGH</i>	BA	<i>SpeI</i> -F	6.8
<i>rmHG</i>	GA'	<i>SpeI</i> -AA	10.0
<i>rmDE</i>	CG	<i>SpeI</i> -EE	10.1
<i>rmCA</i> (left arm)	C	<i>BlnI</i> -TU	9.9
<i>rmCA</i> (left arm)	C	<i>XbaI</i> -DD	9.9
<i>rmCA</i> (right arm)	E	<i>BlnI</i> -V	6.5
<i>rmB</i>	EF	<i>XbaI</i> -EE	13.2
<i>rmCA</i> (right arm)	E		6.5
<i>rmEC</i> (left arm)	F		6.0
<i>rmCA</i>	CE	<i>SpeI</i> -FF	17.4
<i>rmB</i>	EF		13.2
<i>rmEC</i>	FD		10.6
<i>rmB</i>	EF	<i>BlnI</i> -X	13.2
<i>rmEC</i> (left arm)	F		6.0
<i>rmEC</i> (right arm)	D	<i>BlnI</i> -Y	5.8
<i>rmAD</i>	DB	<i>BlnI</i> -Z	12.5
<i>rmAD</i>	DB	<i>SpeI</i> -GG	12.5

^a The *rm* operons were isolated from *S. typhi* Ty2, which is of GT9 and RT5, as illustrated in Fig. 1B. The restriction fragments and *rm* operons were identified to Fig. 1 in reference 12.

between the two halves. The GT detected by partial I-CeuI digestion will detect most changes of order of the I-CeuI fragment but will not detect inversions of I-CeuI-A (due to recombination between *rmH* and *rmG*) or inversions of I-CeuI-C (due to recombinations between *rmD* and *rmC*). Thus, the RTs of 55 of the 57 strains of GT3 can be explained as being due to recombination between *rmH* and *rmG*; there is no need to invoke mutation in *PstI* sites to explain the occurrence of these strains. However, there may be a minor role for mutation in the *PstI* sites in producing new RTs among strains in GT3; the sizes of fragments in one strain of RT3 and one of RT4 could not be explained by recombination alone.

(ii) A further indication that new RTs result from the genomic rearrangements which produce new GTs is the observation that strains with different GTs almost always show different RTs (Fig. 3; Table 1).

Researchers working on ribotyping in *S. typhi* have focused mainly on discriminating among different strains; they assumed that point mutations lead to RT changes (19) or did not discuss the genetic basis (1, 20). Karaolis et al. (8), investigating ribotyping in *Vibrio* spp., assumed that point mutations lead to RT changes and used the frequency of RT changes to calculate the frequency of overall genomic point mutation.

The method of partial I-CeuI digestion will detect genomic rearrangements due to recombination between parts of the *rm* operons and will reveal the order of fragments, thus determining the "rm skeleton" of the genome, i.e., the number of *rm*

operons, and the lengths of the DNA intervals between each of these operons. This is a very efficient method for detecting changes in the genome, either rearrangements of the existing DNA or addition or deletion of DNA (indels). However, this method will not detect inversions of fragments I-CeuI-C or I-CeuI-A in *Salmonella* (or equivalent changes in other genomes). For example, strains of GT3, resulting from homologous recombination between *rmH* and *rmG* to produce *rmHG* and *rmGH*, as shown in Fig. 1C and D, cannot be distinguished by the partial I-CeuI digestion method. Ribotyping, on the other hand, will detect new RTs which result either from recombination between any of the *rm* operons (without the limits that apply to the I-CeuI partial-digestion method) or from mutation in the endonuclease target sites. For simply revealing distinct types, ribotyping is superior to genome typing because it is somewhat more discriminating, since it can distinguish between strains with inversions in I-CeuI fragments A and C (Fig. 1 and 2) and can also detect mutations in the *PstI* sites. However, ribotyping alone will not determine the basis for the new RTs unless the analysis is coupled with partial I-CeuI digestion, as in the analysis we present here.

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