Polymorphism and gene conversion of the 16S rRNA genes in the multiple rRNA operons of *Vibrio parahaemolyticus*

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Abstract

The genome sequence of a strain of *Vibrio parahaemolyticus* holds 11 copies of rRNA operons (*rrn*) with identical 16S rRNA genes (*rrs*). Conversely, the species type strain contains two *rrs* classes differing in 10 nucleotide sites within a short segment of 25 bp. Furthermore, we show here that the sequence of this particular segment largely differs between some strains of this species. We also show that of the eleven *rrn* operons in the species type strain, seven contain one *rrs* class and four the other, indicating gene conversion. Our results support the hypothesis that the *rrs* differences observed between strains of this species were caused by lateral transfer of an *rrs* segment and subsequent conversion.

Keywords: Gene conversion; Polymorphism; *rrn* operons; *Vibrio parahaemolyticus*; 16S rRNA

1. Introduction

Vibrio parahaemolyticus is a natural inhabitant of coastal waters and one of the major seafood-borne gastroenteritis-causing bacteria. Since 1996 an increasing number of *V. parahaemolyticus* infections caused by strains belonging to a clonal complex have been observed throughout the world [1]. We have recently found that a strain of this complex caused two large diarrhoea outbreaks in Chile, in 1998 and 2004 [2]. The genome of one of these strains, RIMD2210633 (VpKX), consists of two circular chromosomes with 11 copies of rRNA operons (*rrn*), 10 on chromosome 1 and 1 on chromosome 2 [3]. Analysis of the reported sequence [3] shows that this strain contains almost identical 16S rRNA genes (*rrs*) in their 11 rRNA operons. However, like many strains which have their genome sequenced [4], most strains of the genus *Vibrio* show detectable sequence differences between their multiple *rrs*. The type strain of the *V. parahaemolyticus* species, ATCC 17802 (VpD), contains two *rrs* classes, differing in 10 nucleotide sites of a 25 bp sequence that encodes a variable stem loop of the 16S rRNA, including nucleotides 440–496 (*Escherichia coli* numbering) [5]. Furthermore, the sequence of each of the segments in the two *rrs* classes of the type strain differ in 7 and 10 nucleotide sites from the corresponding segment in the VpKX strain (see sequences for VpD1, VpD2 and VpKX in Fig. 1). The presence of two different *rrs* classes in the genome of the type strain and the difference between both *rrs* classes of this strain with that in the pandemic strain do not have a simple explanation.

The large number of mismatches together with the compensating changes observed between the two *rrs* segments found in VpD, implies that their divergence

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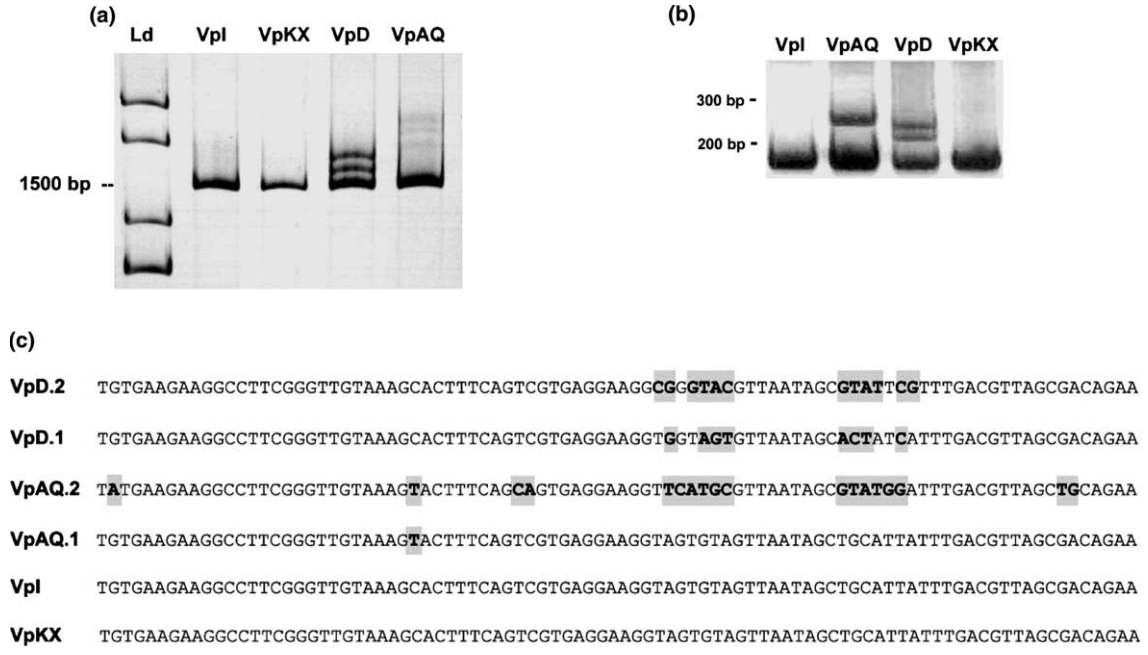

Fig. 1. Polymorphism and sequence differences of the *rrs* in four *V. parahaemolyticus* strains. (a) Polyacrylamide gel electrophoresis of the amplification products of the *rrs* from position 27 to 1492 (*E. coli* numbering). Ld corresponds to the molecular marker ladder, arrows indicate product size in base pairs. (b) Polyacrylamide gel electrophoresis of the amplification products of the *rrs* from position 357 to 518 (*E. coli* numbering). (c) Nucleotide sequences of the *rrs* from position 406 to 496 (*E. coli* numbering), sites showing mismatches are indicated in bold within a shadowed box. The strains are: VpD, species type ATCC 17802^T; VpI, serotype O4:K12 WP-1 or RIMD2210086; VpAQ serotype O3:K6 (1991) AQ4673 or RIMD2210856; and VpKX serotype O3:K6 (1996) KXV237 or RIMD2210633.

is relatively ancient and it is difficult to accept that they evolved in the same cell. Hence, these segments probably evolved in different strains and one of the two versions was probably acquired by lateral transfer. However, it would be expected that after transfer the *rrs* multigene family would probably be homogenized. In most bacterial species, members of the *rrs* multigene family are homogenized to evolve in a concerted fashion [6,7]. Without concerted evolution, mutations would accumulate in individual *rrs* at a similar rate to that observed between species [7], causing high polymorphism among repeated genes of the same family. It is believed that in prokaryotes the homogenization involves gene conversion [7], a process that causes a segment of DNA to be copied onto another segment of DNA, probably by nonreciprocal recombination between genes in the *rrn* operons [8]. Homogenization may occur by conversion of the incorporated segment to the autochthonous version or, alternatively, by conversion of the autochthonous segments to the laterally transferred version. To explore the possible existence of *rrs* conversion in VpD, we determined the number of *rrs* containing each segment sequence. Since the possibility that two or more *rrs* might independently acquire the same sequence segment by random mutation is practically zero, the presence of both sequences in more than one gene would be due to gene conversion. Our results can be best explained by the occurrence of *rrs* conversion among the multiple operons of *V. parahaemolyticus*. The evidence for gene conversion supports the hypothesis that differences between *rrs* of close phylogenetically related strains may arise by lateral transfer of a segment even though they contained multiple *rrn* operons.

2. Materials and methods

2.1. Bacterial strains and media

The *V. parahaemolyticus* strains, RIMD 2210856 (VpAQ), 2210633 (VpKX) and 2210086 (VpI) were directly obtained from the Research Institute for Microbial Diseases, Osaka University, Japan (RIMD) respective culture collection. *V. parahaemolyticus* strain ATCC17802^T (VpD) was directly obtained from the American Type Culture Collection, Manasas, VA. Bacterial strains were grown in Marine Broth (Difco) at 37 °C. The identification of these cultures was confirmed subsequently by the determination of 16S–23S rDNA spacer patterns.

2.2. DNA extraction, PCR amplification

Bacterial DNAs were extracted from overnight cultures [12] and PCR amplifications were performed as previously described [9]. Primers employed for the different

amplification protocols were: Eubac27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) to amplify 16S rDNA, and primers 357F (5′ CTCCTACGGGAGGCAGCA-3′) and 518R (5′-CGTATTACCGCGGCTGCTGG-3′) to amplify the shorter fragment containing the variable region G1F (5′-GAAGTCGTAACAAGG-3′) and L1R (5′-AAGGCATCCACCGT-3′) to amplify the 16S–23S rDNA genes spacer [10]. PCR products were electrophoresed and visualized as previously described [11].

2.3. Cloning and sequencing of rrs together with adjacent spacer

Primers 357F and L1R were employed to amplify the fragments containing both the 16S rDNA fragment and the spacer. PCR products were purified using the Wizard system as indicated by the manufacturer (Promega) and later cloned into pGEMT Easy Vector Systems according to manufacturer's instructions (Promega). Plasmid DNA was obtained by a rapid alkaline extraction miniprep [12]. For analysis of the size of the spacers and the class of *rrs* by heteroduplex assay, the plasmid DNA was diluted 1:100 (vol./vol.) in sterile distilled water and 15 μl were used for PCR amplification of either the 16S rDNA or the spacer, as described above. The size of the spacer was subsequently determined by polyacrylamide gel electrophoresis. The heteroduplex assay was performed as described [13], except that the electrophoresis was conducted at 150 V. For sequencing, plasmids were purified with E.Z.N.A. Plasmid Miniprep Kit I (Omega Bio-tek) and the cloned segments were sequenced on an ABI 3100 Genetic Analyzer using Big Dye Terminator Cycle Sequencing V2.0 Ready Reactions Kit and recommended protocols with primers M13F, 518R, G1F and L1R. DNA sequences were inspected individually and manually assembled. The alignments and sequence similarities were obtained using BioEdit [14].

2.4. Pulsed-field gel electrophoresis and fragment analysis

Bacterial genomic DNA in agarose plugs was prepared as described [15], and digested with the restriction enzyme I-*Ceu*I (New England Biolabs) for 16 h at 37 °C, using 50 U/plug. Electrophoresis was performed on a CHEF DRII System (BioRad,), using a 1% low melting point agarose (Promega) gel in 0.5× TBE buffer (0.45 mM Tris–borate, 1 mM EDTA-Na, pH 8.0). The pulsed time employed was 6–60 s ramp time at 200 V for 24 h, at a constant temperature of 14 °C. After electrophoresis, the gel was stained with ethidium bromide for 30 min and photographed. The observed bands were excised from the gel with sterile razors and a slice of each band was then melted at 65 °C in 10 times its volume of 1× TE (10 mM Tris–Cl, 1 mM EDTA-Na, pH 8.0). Twelve liters of the solution containing DNA from each band was then used for PCR, as described above except that only 20 cycles were performed. Analysis of both the size of the spacers and the class of *rrs* by heteroduplex assay was performed as described above.

2.5. Nucleotide sequences

Sequences have been deposited in GenBank under the Accession Nos. AY298793, AY298798, AY298799–AY298808, and AY527386–AY52735388.

3. Results

3.1. rrs polymorphism in the multi rrn operons of V. parahaemolyticus strains

The polymorphism in the repeated *rrs* of the *V. parahaemolyticus* species type strain was originally observed by the formation of heteroduplexes after PCR amplification of the *rrs* [5]. The presence of heteroduplexes after PCR amplification of a single isolate with multiple *rrs* occurs when these genes exhibit differences in the nucleotide sequences. In polymorphic strains, hybrids between synthesized copies with different sequences are formed, which show a retarded electrophoretic migration in polyacrylamide gels. This assay was employed for assessment of polymorphism in other *V. parahaemolyticus* strains, including the pandemic strain O3:K6 (VpKX), whose genome sequence shows identical *rrs* genes [3]. Fig. 1(a) shows the results obtained after PCR amplification of the 16S rRNA gene from the species type strain VpD, the sequenced pandemic clone VpKX and two other non-pandemic strains; VpAQ, an O3:K6 isolate obtained in 1991, and VpI, an O4:K12 isolate found in 1968. After amplification of the 16S rRNA gene, heteroduplexes with retarded migration were observed for strains VpD and VpAQ, but not for VpKX and VpI. The heteroduplex nature of the bands with retarded migration was confirmed as previously described [13] (results not shown).

Cloning and sequencing of the *rrs* in VpD has shown the presence of two classes, called 1 and 2, which differ in 10 nucleotide positions within a 25 bp segment in a variable stem loop of the 16S rRNA, including nucleotides 440–496 (*E. coli* numbering) [5].To examine if the polymophism observed in VpAQ occurred in this same region, a shorter fragment of 161 bp encompassing the variable segment was amplified by PCR and checked for formation of heteroduplexes (Fig. 1(b)). The presence of extra bands observed above the main amplification products in VpAQ and VpD indicated that the polymorphism was in the same *rrs* region. The presence of two bands above the main band in VpD is probably

due to the differential migration of the reciprocal hybrids formed in this strain. Two different heteroduplexes, composed by plus and minus complementary strands of each amplicon with different sequence, are formed after annealing in the last PCR cycle. Although the extent of dissimilarity in this hybrid pair is the same, non-paired regions may form distinct structural conformations in each hybrid, decreasing the mobility to different extents [5,13,16]. The sequences of the *rrs* variable region in the different strains were determined after amplification and cloning as described below. Fig. 1(c) shows that the sequences found in both VpKX and VpI are identical but they greatly differ from the two sequences found in the species type strain VpD and from one of the two sequences found in VpAQ.

3.2. *Operons with each* rrs *sequence in the species type strain*

To explore if a new sequence may be enforced in every repeated gene, we looked for evidence of gene conversion. Homogenization of the *rrs* requires that one of the versions convert the others. The occurrence of *rrs* gene conversion was explored in VpD. The presence of both sequence versions in more than one operon would indicate gene conversion because the possibility that two or more *rrs* might acquire the same segment sequence by random mutation is practically zero. The sequence of the variable segment of the *rrs* in the different operons of VpD was determined by analysis of restriction fragments obtained by cleavage of the genomic DNA with the restriction enzyme I-*Ceu*I. This enzyme cleaves a 19-bp sequence in the 23S rRNA gene [17] and allows for the separation of the *rrn* operons by gel electrophoresis. Fig. 2(a) shows the result of the pulsed-field gel electrophoresis with the resolved bands numbered from 1 to 8. The sequence of *rrs* (class 1 or 2) is shown to the right of each band. The *rrs* class was defined by a heteroduplex assay based on the retarded migration of the hybrids formed between *rrs* segments of different nucleotide sequence. For this assay, a 161 bp segment containing the *rrs* variable region was amplified from each fragment and the product was hybridized with those obtained from recombinant plasmids containing *rrs* of either class 1 or 2 [5]. Analysis of the hybridization products by gel electrophoresis shows exclusively homoduplexes when amplicons are of the same class, and homoduplex plus heteroduplexes with retarded

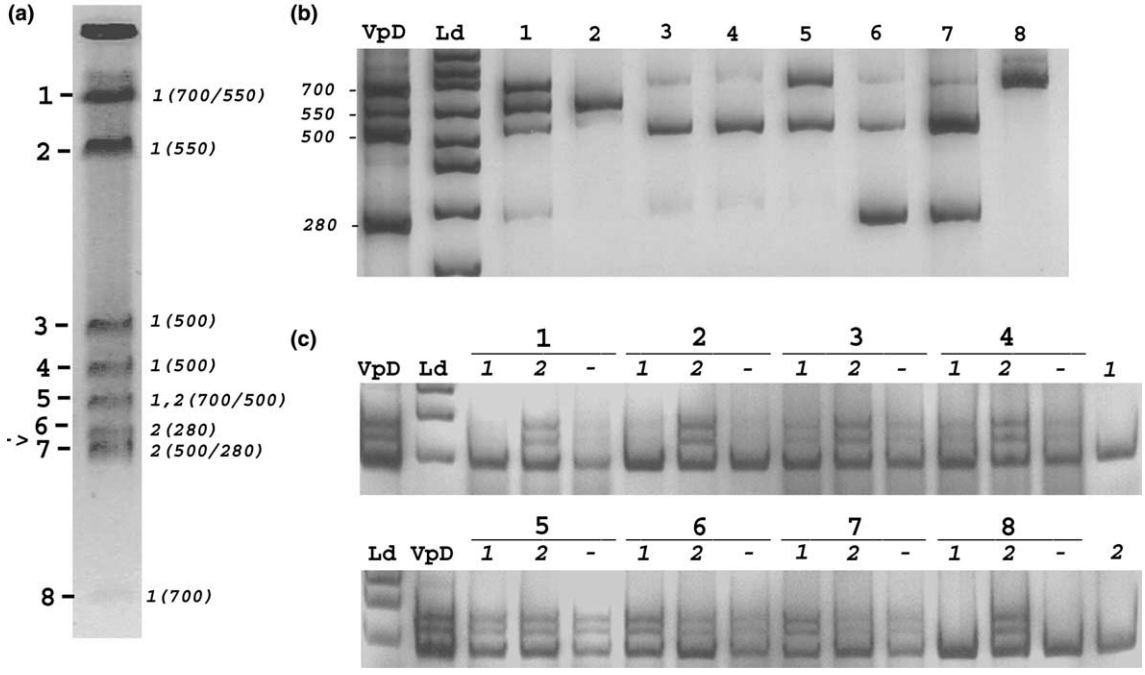

Fig. 2. Separation of the genomic fragments containing *rrn* operons and characterization of their *rrs* genes and *rrs–rrl* spacer regions in *V. parahaemolyticus* strain ATCC 17802. (a) Pulsed-field gel electrophoresis of the DNA digested with I-*Ceu*I. The number assigned to each band is indicated on the left side; the class of *rrs* and the approximate size of the spacers (between parenthesis), observed in each DNA band, are indicated on the right side. (b) Polyacrylamide gel electrophoresis of the *rrs–rrl* spacer regions PCR amplified as described in Materials and Methods. The number on top of each lane indicates the analyzed band. VpD corresponds to the amplification product from the whole DNA of *V. parahaemolyticus* ATCC17802T. Ld corresponds to a 100 bp molecular size marker. (c) Polyacrylamide gel electrophoresis of the products formed after denaturation and annealing of the amplification products of the variable region of the *rrs* contained in each band (indicated above every three lanes) with the amplification products of clones containing *rrs* class 1, class 2 and with themselves (indicated as 1, 2 and - above each lane, respectively). Numbers 1 and 2 in the last lanes to the right correspond to the self-annealing products from class 1 and 2 *rrs* clones, respectively. VpD and Ld as indicated in b.

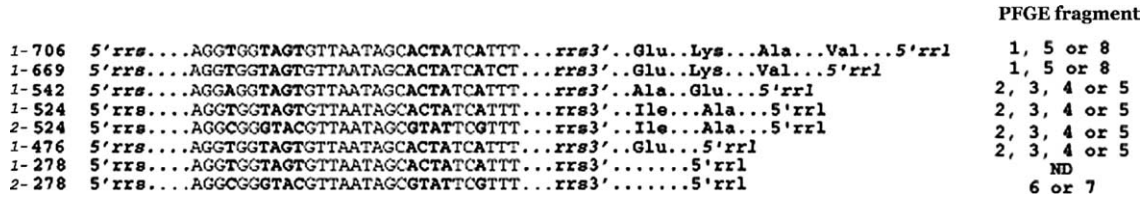

Fig. 3. Schematic representation of the different putative operons identified both in the clones of the PCR amplification products and in the restriction fragments. Nucleotides in polymorphic sites are in bold. Each operon is identified by the class of *rrs* (1 or 2) and the size of its neighbor spacer in bp (first column on the left). The scheme shows the sequence of the variable region of the *rrs* followed by the tRNAs present in its adjacent spacer. Pulsed field electrophoresis bands that may contain the operon are identified on the right side. The uncertainty is caused by the inability to distinguish between spacers of almost equal size by gel electrophoresis, i.e., 706 and 669. ND, not detected among the pulsed field gel electrophoresis bands.

migration when the amplicons are of a different class. Fig. 2(c) shows the result of the heteroduplex assay for each band. For some bands the presence of *rrs* of a single sequence class is straightforward, as for the presence of sequence class 1 in bands 1, 2 and 8 or class 2 in band 6 observed in this figure. Band 5 shows the presence of heteroduplexes when hybridized with amplicons from the clones of either class 1 or 2 and with itself. This kind of result is expected when the band contains both *rrs* classes. Heteroduplexes observed in minor proportion after self-annealing of the amplicons of some bands, e.g., band 7, are likely due to contamination in the gel with other restriction fragments.

To define the number of different operons in each band we determined the size of the 16S–23S rDNA spacer regions by PCR amplification. The size of the spacers allows for the placement of the operons of this strain into six groups [18]. Fig. 2(b) shows the spacers observed after amplification of each band; the observation of more than one spacer in some bands (bands 1, 5 and 7) may be due to either the co-migration of two restriction fragments or to the presence of two operons. Fragments with two operons may be generated when two neighbour operons are in opposite direction. The sizes of the spacers found in each band are shown between parenthesis in Fig. 2(a). Altogether, the determination of the size of the spacer and class of *rrs* allowed for the identification of 11 putative rRNA operons, seven containing *rrs* 1 and four *rrs* 2.

The class of *rrs* was also determined on a complementary approach, independent of the location of the *Ceu*I restriction sites. This entailed the amplification and cloning of about three-fourths of the *rrs* genes together with their entire neighbouring spacers. The operons in 44 clones of the product were examined for both the size of the spacer and the class of *rrs*. They were initially separated into four groups according to the estimated size of the 16S–23S rDNA intergenic spacers by gel electrophoresis [18]. According to both the estimated size of the spacer and the class of the *rrs*, six groups of clones were distinguished. At least four clones from each group were sequenced. These sequences permitted the identification of 8 groups of clones; six groups containing *rrs* with sequence class 1 and two with sequence class 2. Fig. 3 shows a scheme of the *rrs* class and the size of the spacer in the different groups. The sequence of the variable region of the *rrs* and the tRNAs deduced from the spacer sequences are schematically shown on the right side. As previously described by Maeda et al. [18], the sequences we found allowed us to distinguish six groups of spacers although only four bands are observed after polyacrylamide electrophoresis.

3.3. Gene conversion in the 16S–23S rDNA spacer regions

To explore gene conversion in other regions of the *rrn* operons, the sequence of the spacer in the different operons was analysed. The analysis of the spacer sequences in VpD showed conserved sequence blocks in each *rrn* operon corresponding to the 40 sites next to the 5′ end and the 208 sites next to the 3′. Though these blocks are highly conserved, a few polymorphic sites suggestive of gene conversion were observed. Within the 40 sites next to the 5′ end, there is a polymorphic site with T in two putative operons and A in the other six. Within the 208 sites next to the 3′ end there is a polymorphic site with T in two putative operons and G in the other six (results not shown).

4. Discussion

The high polymorphism observed between the multiple *rrs* in VpD and VpAQ in a particular segment and the differences between *rrs* of different strains in this same segment is extraordinary. The segments with the large changes found within the same strain are unlikely to be due to accumulation of point mutations. It seems more probable that each segment evolved independently in different strains and that they came together by lateral transfer. This transfer might have occurred via replacement of a segment as proposed by Wang and Zhang [19] in their simplified complexity hypothesis. Interestingly, despite their approximate 40% dissimilarity, the

different versions of the 25 bp segment in the four *V. parahaemolyticus* strains fully match with those in the 16S rRNA of strains of the genus *Vibrio* or with non-classified marine *Vibrionaceae*, exclusively. Most isolates containing any of the sequence versions found in *V. parahaemolyticus* corresponded to the species *V. vulnificus*, *V. coralliilyticus*, *V. alginolyticus*, *V. fischeri*, and *V. ponticus*. Being an autochthonous marine bacterium, *V. parahaemolyticus* is probably subjected to a high level of recombination with the diverse, closely related bacterial strains populating the seawater. Seawater is a particular habitat where vibrios are exposed to high levels of gene transfer by transduction [20]; two bacteriophage classes that could potentially transfer *rrs* genes among Vibrio related strains have been reported. These comprise filamentous phages [21], including one that is able to integrate into the host chromosome of *V. parahaemolyticus* [22,23] and T4-related broad host range phages [24]. However, lateral transfer would probably change only one of the multiple *rrs*. Changing more than one or every *rrs* in the genome would require gene conversion. Our analysis of the *rrn* operons showed the presence of at least 7 operons containing *rrs* with sequence class 1 and class 4 with sequence class 2. Considering the number of different nucleotides together with compensatory mutations required for the generation of the two *rrs* classes, it is very unlikely that the redundant sequences appeared independently in more than one operon. Although the occurrence of reciprocal double exchanges between sister chromosomes, as described by Segall and Roth [25] may generate redundancy of the sequences, it seems more likely that the redundancy described above was caused by gene conversion. The repeated mismatches found in the spacer regions are also suggestive of the occurrence of gene conversion. If gene conversion occurs at a higher rate than gene differentiation, the *rrs* would become identical, as was observed in VpI and VpKX. However, besides having identical segments in their multiple *rrs*, these two strains contain a sequence segment very different from that observed in the species type strain. Therefore, it seems probable that the observed differences between strains may have originated by lateral transfer of the variable segment followed by gene conversion of other *rrs* in the genome. A similar mechanism for the polymorphism and redundancy observed in the intervening sequences of the multiple *rrl* copies present in *Salmonella typhimurium* and *Salmonella typhi* has been postulated by Mattatall et al. [26,27].

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